STUDIES ON THE DEVELOPMENT

OF

STANDARDISED THROMBOPLASTIN REAGENTS

by

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The experimental work reported in this thesis was performed by the author. None has been submitted for any other degree of the Council for National Academic Awards, or of any university.

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SUMMARY

Chapters 1 to 5 provide an introduction, an overview of the blood coagulation mechanism, and reviews of tissue thromboplastin, partial thromboplastin and freeze-drying. Chapter 6 describes materials and methods. In Chapter 7 the structure of the thromboplastin reagent is examined and changes to this structure and to the lipid moiety of the reagent on prolonged storage at $4^{o}C$ are demonstrated. In Chapter 8 the clotting activities, ultrastructure and biochemical composition of several partial thromboplastin reagents are compared. The liposomal nature of the reagents is demonstrated and correlations are established between clotting activity and electrophoretic mobility, liposome size and lipid composition. Liposomes of varying composition are prepared. By adjusting the relative concentration of phosphatidyl serine it is possible not only to regulate procoagulant activity and sensitivity to heparin but also to produce a liposome that accelerates the APTT of plasmas containing a lupus-like inhibitor while having no effect on APTT prolonged by other types of inhibitor, factor deficiencies or oral anticoagulants. In Chapter 9 the freeze-drying of tissue thromboplastin reagents is examined. A freeze-drying sequence is described that achieves a residual moisture of 1.8%, a value that is shown to be acceptable for long-term storage.

Modifications to the freeze-drying machinery are evaluated. It is shown that traditional views on ratio of shelf area to condenser surface area may be incorrect. The freeze-drying machinery is found to be free of any intrinsic variation. A description is given of

the freeze-drying of three International Reference Preparations of thromboplastin and consideration is given to their long-term stability. Finally, a comparison is made of rubber-stoppered glass vials with fusion-sealed vampoules. The latter prove to be more reliable for storage of reagents at high temperature. Chapter 10 contains a summary and conclusions.

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SOME HEALTH AND SAFETY CONSIDERATIONS

Two main classes of hazards encountered during the course of this work were 1) biological and 2) fire. The biological hazards stemmed from handling blood and plasma, both human and non-human, and from human brains which were received unfixed from post mortem rooms. To reduce the possibility of dangers from these materials, they were processed in a room set aside for the purpose, with appropriate extraction systems, by trained staff wearing suitable disposable clothing. Testing of brain extracts for bacteriological contamination and testing of brain extracts and plasma for hepatitis, and HIV when a test became available, was routinely undertaken.

Fire hazards were due to the need for organic solvents for lipid extraction and chromatography. Solvents were stored in locked steel cupboards in a secure outbuilding designed to house inflammable substances. Only the absolute minimum volumes of solvents were taken to the laboratory, on a daily basis, and were stored in metal boxes during this time. A further possible fire hazard was the hydrogen gas required for the flame ioniser detector in the gas chromatograph. The gas was piped to the laboratory from a gas cylinder store, situated in a secure outbuilding. Testing for leaks was a matter of daily routine. Smoking and naked lights were not permitted in the laboratory.

ABBREVIATIONS USED IN THE THESIS

BCR	Bureau Communautaire de Reference
EEC	European Economic Community
WHO	World Health Organisation
ICSH	International Committee for Standards in Haematology
BCT	British Comparative Thromboplastin
MCR	Manchester Comparative Reagent
ISI	International Sensitivity Index
INR	International Normalised Ratio
ICC	International Calibration Constant
ICR	International Calibrated Ratio
PL	phospholipid
PA	phosphatidic acid
PS	phosphatidyl serine
PE	phosphatidyl ethanolamine
PC	phosphatidyl choline
PG	phosphatidyl glycerol
PI	phosphatidyl inositol
SPH	sphingomyelin
lyso-PC	lysophosphatidyl choline
di-PG	di-phosphatidyl glycerol (or cardiolipin)
chol	cholesterol
FFA	free fatty acid(s)
16:0	palmitic acid - 16 is the number of carbon atoms, 0 means that there are no double bonds in the molecule

18:3ω6,9,12	linolenic acid - 18 carbons are to be found in the molecule and 3 double bonds. The "ω" shows that these occur at bonds 6, 9 and 12, counting from the methyl end of the molecule
USFAR	unsaturated to saturated fatty acid ratio
mda	malondialdehyde
v/v	volume to volume ratio
TLC	thin-layer chromatography
Rf	relative to front. A term used in TLC to describe mobility of separated spots. Rf is the ratio of distance from origin to spot/distance from origin to solvent front.
RM	residual moisture
wt	weight

CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1 AIM OF THE STUDY

The aim of the investigations presented in this thesis is to elucidate current understanding of tissue thromboplastin and partial thromboplastin reagents and to improve the preservation of standardised tissue thromboplastin reagents by freeze-drying.

The present author previously described the successful freeze-drying of tissue thromboplastin reagents derived from human brain (Stevenson 1978). The lipid class composition of human brain thromboplastin reagent was determined and an effort was made to determine the changes undergone by the material during storage, in the liquid form at 4°C, and in the lyophilised state at a variety of elevated storage temperatures. Some progress was made in the area of measurement of lipid class composition in these reagents and in the use of protective antioxidant compounds.

This thesis attempts to expand on the work on tissue thromboplastin, by examining further the lipid class composition, including fatty acid distribution and presenting evidence of the structure of tissue thromboplastin reagents, both fresh and aged. Consideration is also given to the technique of freeze-drying of thromboplastin and data is presented to show how changing conditions within the freeze-drying machinery affects the final product. Attention is also given to the freeze-drying containers employed for tissue thromboplastin reagents and it is shown that fusion-sealed glass vampoules provide more reliable vessels for long-term storage of the reagent at elevated temperatures than rubber-stoppered

vials. There is now a recognised system for standardisation of the prothrombin time based on the use of international reference preparations for thromboplastin which has a place in its hierarchy for three thromboplastin reagents which have been prepared at the UK Reference Laboratory for Anticoagulant Reagents and Control.

Unfortunately, no equivalent system yet exists for partial thromboplastin reagents. One reason for this may be the general lack of understanding of the lipid reagent, or cephalin, central to the partial thrombplastin time test system.

Some fundamental questions are addressed, therefore, in this work and it is shown that currently available, widely used partial thromboplastin reagents are comprised of liposomes of varying lipid composition and structure, and that these properties influence the clotting activity of the test systems in which they are used. To test this further, a series of liposomes is prepared which demonstrate the central importance of phosphatidyl serine in the regulation of the procoagulant activity of partial thromboplastin time systems, their sensitivity to heparin and to the detection of the lupus anticoagulant. These observations may be valuable in formulating a lipid reagent that might serve as a reference preparation.

In order to put these experimental data in their context, the introductory chapters offer a view of the haemostatic system, and reviews of the biology of tissue thromboplastin and its role as a laboratory reagent. A review of the events leading to the current

understanding of the partial thromboplastin is then presented, with consideration of the laboratory use of partial thromboplastin reagents. A section is then devoted to freeze-drying, a technique which is incompletely understood, despite its common use. A chapter confined to details of the methods and materials employed opens the experimental section.

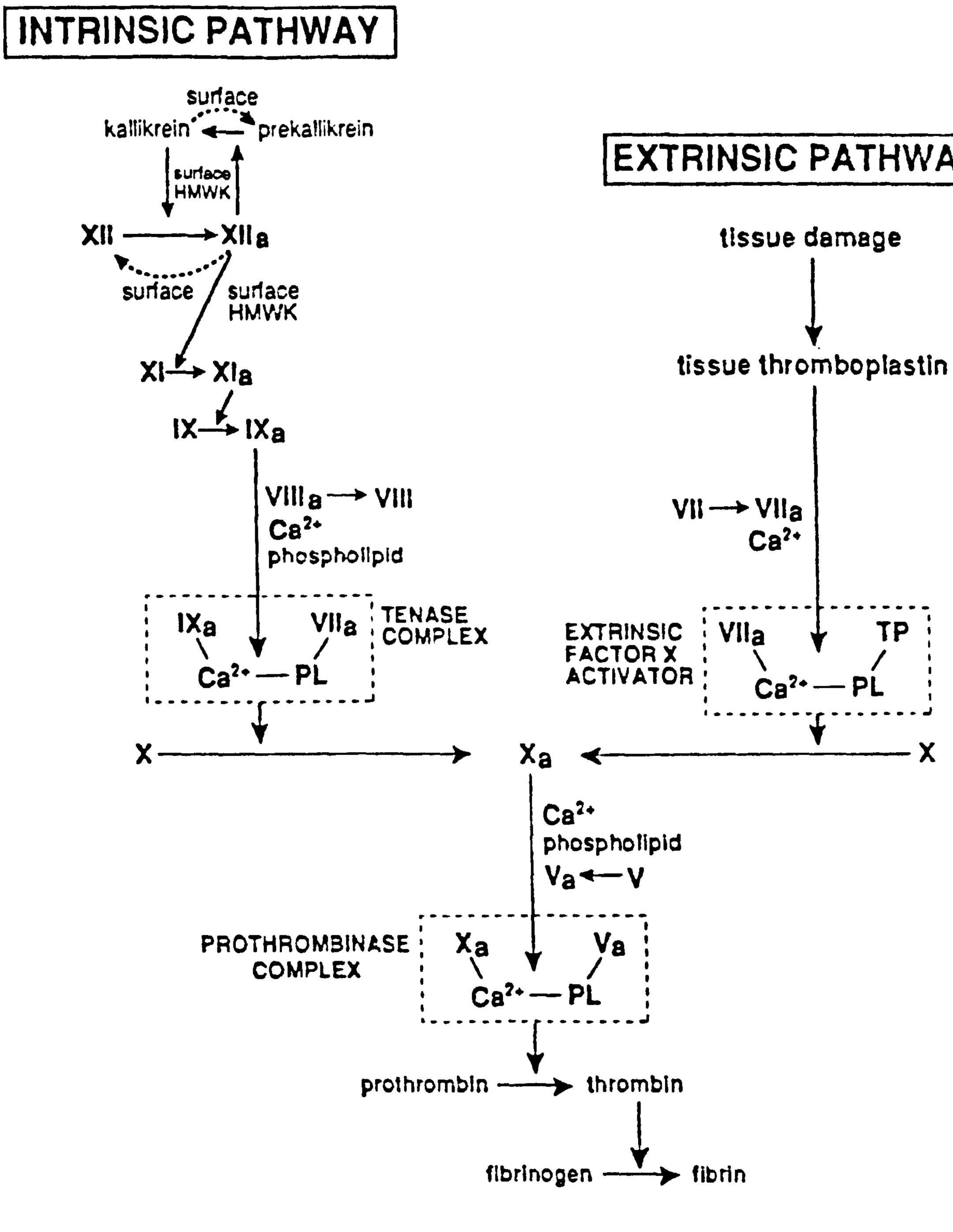
CHAPTER 2 THE MECHANISM OF BLOOD COAGULATION

In the first part of this chapter a limited view of the blood coagulation mechanism is presented and is confined to areas relevant to the subject matter of this thesis. These include the initiation of the sequence by tissue thromboplastin, the form of phospholipids and biological membranes and their role at several stages of the process. The second part of the chapter describes some anticoagulant mechanisms and examines the effect of dicoumarol on the biosynthesis of clotting factors, the roles of protein C and protein S, and the action of heparin.

2.1 THE HAEMOSTATIC MECHANISM - THE ROLES OF TISSUE THROMBOPLASTIN AND PHOSPHOLIPIDS

Traditional Theories of Blood Coagulation

Traditionally, the blood coagulation mechanism was considered to be a "cascade" (MacFarlane 1964) or "waterfall" (Davie and Ratnoff 1964) of plasma proenzymes to their active enzyme forms, leading ultimately to the formation of fibrin. This cascade was depicted as having an "intrinsic" pathway and an "extrinsic" pathway resulting in the activation of factor X. In the former, no agent external to the blood was considered necessary while the latter depended upon an ill-defined interaction of tissue thromboplastin with factor VII to activate factor X. Fig 2.1 shows the classical scheme with its clear separation of intrinsic and extrinsic pathways.



HMWK	high molecular weight kininogen
PL	phospholipid
Ca^{2+}	calcium ions

Fig 2.1 A scheme of blood coagulation.

While the current view of the haemostatic mechanism inevitably requires some modification to this scheme, the most important premise of the classical system is unaltered i.e. the idea of the activation of proenzymes to active enzymes by limited proteolysis. This elegant control mechanism is at the heart of the system.

Prothrombin, for example, may be activated by a single proteolytic cleavage. Prothrombin is a single chain glycoprotein with a molecular weight of 60,000 daltons, to be found in plasma at a concentration of 100 $\mu g\ cm^{-3}$. This first cleavage produces meizothrombin, which is a two-chain molecule, because the two parts of the originial molecule resulting from the cleavage remain attached via a disulphide linkage. This molecule has the capacity to split small peptides in the same way as thrombin, but before it can carry out the role of thrombin in coagulation, a second cleavage is required. This cleavage separates thrombin from a large activation peptide known as fragment 1,2 (Owen et al 1974, Esmon and Jackson 1974, Esmon et al 1974). The action of thrombin on clotting factors V, VIII, XIII and fibrinogen, on platelets, on protein C, and on endothelial cells cannot be mimicked by meizothrombin, (Nesheim and Mann 1979, Suzuki et al 1982) showing that not only is an active site required for enzymatic activity, but also a correct arrangement of binding sites. This is an important means of regulation of the reaction sequence.

Pathways involving Tissue Thromboplastin

Tissue thromboplastin is discussed at length in Chapter 3. Many years of research have gone into the current understanding of how this protein, also known as tissue factor, interacts with the blood coagulation sequence. Hjort (1957) suggested that a product of the reaction of factor VII, tissue factor and calcium was responsible for extrinsic activation of factor X while Hougie (1959) believed factor VII had enzymatic properties and that factor X was its natural substrate. Recently, Bom and Bertina (1985) showed that factor VIIa in the absence of any co-factors can activate factor X. Addition of calcium ions stimulated the reaction rate eight-fold, and phospholipids, in the form of a liposome comprising phosphatidyl choline (PC) and phosphatidyl serine (PS) in 1:1 ratio, resulted in a further 250-fold stimulation. They attributed this increase to an accumulation of enzyme and substrate in the phospholipid membrane. The addition of tissue factor to this system resulted in a 20,000 -fold stimulation in the rate of factor X activation.

Factor X is a two chain proenzyme to be found in the circulation at a concentration of 10 $\mu g\ cm^{-3}$. Its molecular weight is 56,000 daltons. Factor VII is a single chain molecule with a molecular weight of 50,000 daltons and is present in plasma at a concentration of 0.5 $\mu g\ cm^{-3}$. These two factors, together with factor IX and protein C show marked homology in structure (Furie and Furie 1988).

It has been shown by several groups that there is little difference in the affinity of tissue factor for binding to factor VII or factor VIIa (Bach et al 1986, Zur et al 1982, Broze 1982). Zur and his colleagues calculated that the proenzyme factor VII had about 0.8% of the activity of the two chain factor VIIa. This was confirmed by Jesty and Morrison (1983). The initial event in the extrinsic factor X activation is probably the formation of a complex of factor VII and tissue factor. This catalyses the activation of factor X to factor Xa which can then cleave factor VII to factor VIIa. Nemerson and Gentry (1986) proposed an ordered addition model for the assembly of the catalytic complex in which factor VIIa undergoes two separate conformational changes. The first of these was due to binding of tissue factor and the second due to the binding of factor X to the factor VIIa-tissue factor complex. This second change induced a 100-fold increase in the affinity of factor VIIa for tissue factor and prevented the dissociation of factor VIIa from the complex.

In 1977, Osterud and Rapaport showed that the factor VIIa-tissue factor complex was able to activate factor IX. Until this time it was believed that factor IX could be activated only by factor XIa, via the intrinsic pathway. Jesty and Morrison (1983) and Sanders et al (1985) observed a lag in the activation of factor IX by factor VII-tissue factor, confirming that factor IXa is also an activator of the proenzyme factor VII.

It may be seen, therefore, that the tissue factor-factor VII complex can contribute to the activation of factor X in two ways,

either directly, or via factor IX and factor IXa. Van Dieijen et al (1981) and Mertens et al (1985) showed that the latter reaction depends on the availability of factor VIIIa. Factor IX shares many common features with factors X and VII, and protein C. It has a molecular weight of 56 000 daltons and is to be found in plasma at a concentration of 5 $\mu g\ cm^{-3}$.

A number of workers have concerned themselves with discovering the relative contribution of the two possible pathways of factor X activation. It is well known that patients with severe deficiencies of factors XI or XII have only a very mild bleeding tendency when compared to patients with severe factor VIII or IX deficiencies. This could indicate that coagulation in the normal physiology depends very little both on the intrinsic pathway (the pathway that proceeds via factor XI - IX - X) and on the direct factor X activation pathway described above (the VII - X pathway). Bertina et al (1981) have described a patient with an abnormal factor X molecule (factor X Utrecht) which can be activated by the factor IXa - VIII complex, but not by the factor VII-tissue factor complex. The patient had a prolonged prothrombin time and a normal partial thromboplastin time and had never suffered a bleeding diathesis. A similar abnormality has been observed by Girolami et al (1985) in a patient also free from bleeding symptoms.

A different line of evidence comes from transfusion experiments in normal and haemophilic (factor VIII deficient) dogs. Mertens et al (1985) found that the infusion of purified factor VIIa in haemophilic dogs did not result in increased fibrinopeptide A

levels, while similar infusion in normal dogs resulted in an approximately 100-fold increase in plasma fibrinopeptide A. These experiments offer strong evidence that the direct activation of factor X by the factor VII-tissue factor complex is of only minor importance.

Hemker (1988) discussed the kinetic efficiency of these various pathways. He points out that several mechanisms exist to remove thrombin from the coagulation sequence e.g. antithrombins will inactivate it while diffusion will transport it away from the site of tissue damage. It is essential, therefore, that a minimum concentration of thrombin must be produced for successful propagation of the clotting sequence. A three-step enzyme cascade will eventually provide sufficient thrombin (Hemker and Hemker 1969) but if thromboplastin is in short supply a four-step cascade will be more efficient. This is provided by the activation of factor IX by VIIa-tissue thromboplastin complex, (Josso and Prou-Wartelle 1956, Marlar and Griffin 1981, Osterud and Rapaport 1977) which in turn activates factor X and so on. It may be significant that haemophiliacs tend to bleed in thromboplastin-poor organs such as skin, joints and muscle while bleeding in thromboplastin-rich organs, such as brain and lung is much rarer (van Trotsenburg 1975, Drake et al 1989). Because the activation of factor IX is slower than that of factor X, the reinforcement loop, provided by factor IX activation, does not contribute significantly to the reaction velocity in the prothrombin time test. For this reason, haemophiliacs have a normal prothrombin time (Hemker 1988).

He also points out that the classical description of the coagulation mechanism is based on some unrealistic premises e.g. the clotting of blood in the absence of thromboplastin, initiated by glass, kaolin, ellagic acid etc. In this system the contact factors interact after absorption to a "foreign" surface and this interaction results in the formation of factor XIa, which can activate factor IX.

The extrinsic pathway was envisaged as the activation of factor X by factor VII acting in concert with tissue thromboplastin, which was held to be external to the circulation. As Hemker (1988) reminds us, foreign surfaces are rarely encountered unless artificial organs and extracorporeal circulation are employed and an excess of thromboplastin will be equally rare unless massive damage occurs to brain, lung or placenta. The most likely physiological activator of clotting, therefore, is a small amount of tissue thromboplastin.

When tissue thromboplastin is exposed to the blood it will bind factor VII and subsequently activate trace amounts of factors X and IX at about similar rates. The formed factor Xa will rapidly activate factors VII, VIII and prothrombin. Factor X activation will continue by the catalytic action of both the factor VIIa-tissue factor complex and the factor IXa-VIIIa complex. The rate of the first reaction will be efficiently suppressed by the inhibition of the factor VIIa-tissue factor pathway by factor Xa, while the rate of the second reaction is controlled by the availability of VIIIa and will continue until the factor VIIIa has been inactivated, for

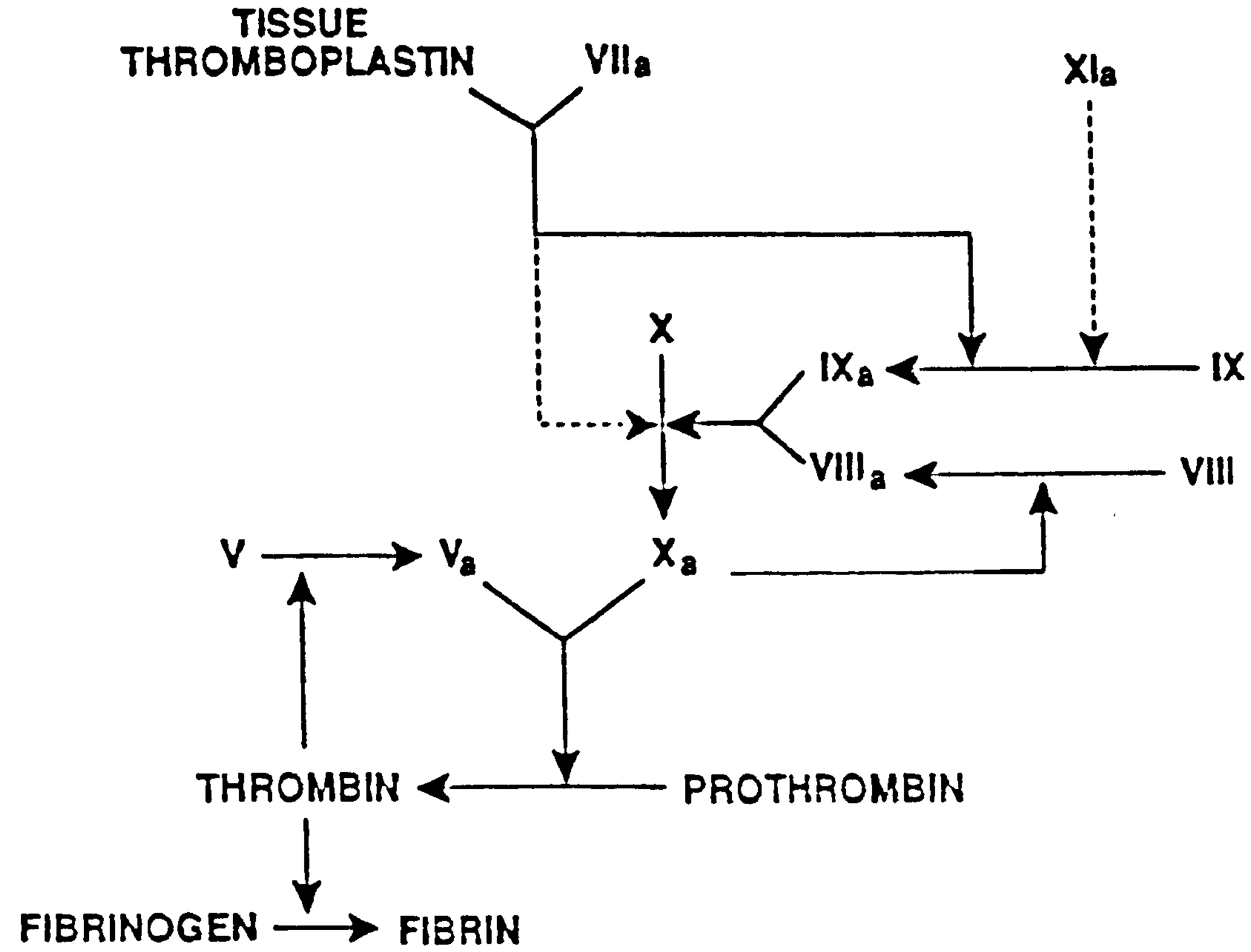


Fig 2.2 An alternative scheme of blood coagulation.

(from Bertina et al. 1988)

instance by the proteolytic action of the activated protein C-protein S complex (Clouse and Comp 1986). The scheme, as described by Bertina et al (1988) is shown in figure 2.2.

Lipids and Biological Membranes

Gorter and Grendel (1925) proposed a model of the biological membrane based on a lipid bilayer. Their suggestion provided the basis for modern theories. The "fluid mosaic" model described by Singer and Nicolson (1972) is now well accepted, providing a working model which accommodates a wide variety of plasma membrane phenomena. The model retains the lipid bilayer concept, both surfaces being depicted as largely naked lipid. Proteins are visualised as being embedded in the bilayer, going part of the way through, in the case of so-called extrinsic proteins, or all the way through in the case of intrinsic or integral proteins. The tissue factor protein is an example of this latter group (see Chapter 3).

It is the amphipathic nature of the membrane lipids that governs their arrangement in cells or in model biomembrane systems i.e. they possess a highly polar group at one end, attached to a hydrophobic tail. The majority of the membrane lipids are in the form of glycero-phospholipids which have a glycerol molecule as their common structural feature. Fatty acids are esterified to the hydroxyl groups at positions 1 and 2 while the hydroxyl group at position 3 is esterified to phosphate. The simplest of the phospholipids is therefore 1,2-diacyl-sn-glycero-3-phosphate which is usually known as phosphatidic acid and abbreviated to PA. This

molecule will carry a strong negative charge at physiological pH. The phosphate group is esterified to either ethanolamine, choline or serine to give, respectively, 1,2-diacyl-sn-glycero-3-phosphoryl ethanolamine,-choline or -serine. These are known as phosphatidyl ethanolamine (PE), -choline (PC) or -serine (PS). PS will be negatively charged at physiological pH due to its possession of a carboxyl group. The NH_2 group will be protonated but will be compensated for by the negative charge on the phosphate moiety. PC and PE each have an NH_2 and a phosphate group and will therefore be zwitterionic and neutral at physiological pH. 1,2-diacyl-sn-glycero-3-phospho-inositol, known as phosphatidyl inositol (PI), and two phosphorylated derivatives, phosphatidyl inositol-4-phosphate (PIP) and -4,5-bisphosphate (PIP_2) comprise approximately 2 or 3 percent of the total phospholipid in plasma membranes. These are highly negatively charged molecules, as are phosphatidyl glycerol (PG) and diphosphatidyl glycerol (di-PG) or cardiolipin.

Another major phospholipid component of plasma membranes is sphingomyelin (SPH). In this molecule, the polar headgroup is phosphocholine, as in PC, but the hydrophobic region is formed by the aliphatic tail of sphingosine plus a long chain fatty acid, which is connected to the NH_2 group of the sphingosine by an amide bond.

Cullis and de Kruiyff (1979) have offered an explanation of how the chemical structure of individual phospholipids determines the structure assumed when the molecule is hydrated. The average

diameter of the polar headgroup of a lysophospholipid is larger than that of a single fatty acyl chain (a lysophospholipid is a phospholipid which contains only one fatty acid). The molecule therefore will have a cone shape, with the polar group at the non-pointed end. As a result, lysophospholipids will form micellar structures. PC and SPH, on the other hand, have a cylindrical shape which will form bilayers, irrespective of temperature, pH, ionic strength or the presence of calcium ions. Providing the buffer is not acidic and is calcium-free, bilayers will also be formed by PS, PI, PG, PA and di-PG. At acidic pH or in the presence of calcium ions, however, the bilayers may assume the hexagonal (H_{II}) phase consisting of a hexagonally packed cylinder with the polar headgroup on the inside. PE, when it contains unsaturated fatty acids may also tend to form hexagonal phase, due to the relatively small size of the head group giving a cone shape to the molecule when attached to the relatively large diameter unsaturated fatty acids.

Cullis and Verklej (1979) have shown that in the absence of calcium, PS stabilizes the bilayer structure. The addition of calcium induces phase separation of PS, allowing PE to form the hexagonal phase. At the same time extensive fusion between bilayers occurs. This process is similar to the process thought to occur during the secretory event described by Satir (1975) e.g. in activated platelets, where an influx of calcium is required to trigger the release of granules into the extra-cellular environment.

In those membrane phenomena which are impossible to explain in terms of a strict, unchanging bilayer model, it may be that

transient formation of non-bilayer phases is recognised as a lesion by the immune system and that the anti-phospholipid antibodies reported in systemic lupus erythematosis result from this phase change (Rauch et al 1986). The form of hydrated phospholipids and its significance in the APTT test is discussed further in Chapter 8.14.

The fatty acyl constituents of phospholipids, therefore, are highly significant in determining the physical character of the molecule and, thereby, fundamental membrane characteristics, for example fluidity (Cullis et al 1985). The great variety to be found in the fatty acid constituents, e.g. in chain length and degree of unsaturation, means that over twenty molecular species may occur within each phospholipid class. An alternative to fatty acids in the hydrophobic moiety of a glycerophospholipid may be offered by fatty aldehydes, which attach by a vinyl ether linkage to the 1 position of the glycerol backbone to form a plasmalogen. The composition of phospholipids in human and rabbit brains is broadly very similar with the notable exception of the ethanolamine containing portion. In the human some 36% of the total phospholipid is in the form of diacyl phosphatidyl ethanolamine, while in the rabbit about 13% of the total is diacyl PE and 23% is plasmalogen (White 1973).

Implicit in the fluid mosaic model is the idea of an extreme degree of fluidity. The term fluidity embraces several distinct features of the lipid bilayer - one type of fluidity involves motion of the hydrocarbon chain relative to the head group. In the gel

conformation the hydrocarbon chains show little mobility. This state is referred to as ordered or crystalline and the abbreviation $L\beta$ is used to indicate a lamellar arrangement in gel conformation. In the liquid crystalline, or random, conformation the hydrocarbon chains are highly mobile and the term $L\alpha$ is used to indicate a lamellar arrangement in this state. Raising the temperature can form $L\alpha$ from $L\beta$ and the temperature at which this conformational change takes place is known as the transition temperature (Tc). The Tc will depend upon the nature of the head group and on the length and degree of unsaturation of the hydrocarbon chains. Phase transition from $L\beta$ to $L\alpha$ is accompanied by an increase in bilayer thickness and in area per lipid molecule (Chapman et al 1967, Phillips and Chapman 1968) as well as an approximately ten-fold increase in lateral diffusion of phospholipids.

Van Deenen et al (1962) and Demel et al (1976) showed that cholesterol has a condensing effect on phospholipids in bilayers, decreasing the average area per molecule. When cholesterol is introduced into the bilayer, the steroid rings interact with, and partly immobilize, those regions of the hydrocarbon chains closest to the polar head group, while leaving the rest of the chain flexible. As a result, cholesterol abolishes temperature-induced phase transition and imparts an intermediate fluid condition to the interior of the bilayer.

A further sense of the term fluidity refers to the lateral diffusion of lipid molecules within one half of the bilayer. At body temperature, natural lipid molecules may exchange places with

their neighbours more than a million times per second (Zwaal and Hemker 1982). Exchange across the bilayer, known as flip-flop translocation, on the other hand, occurs infrequently unless bilayer integrity is disturbed. Van Meer and Op den Kamp (1982) and Middelkoop et al (1986) have shown that species of PC containing one or two unsaturated fatty acids experience transbilayer movements at higher rates than saturated species e.g. 1,2-dipalmitoyl-PC showed a flip-flop half time of about 26 hours compared to approximately 14 hours for 1,2-dioleoyl-PC.

Overall unsaturation, however, was not the determining factor, since the rate for 1-palmitoyl,2-linoleoyl-PC was about five times as fast as that of 1,2-dioleoyl-PC i.e. 2.9 hours compared to 14.4 hours. 1-palmitoyl,2-arachidonyl-PC was three times slower than 1-palmitoyl,2-linoleoyl-PC, again demonstrating that degree of unsaturation is not strictly proportional to rate of flip-flop. These experiments were carried out in the presence of lipid transfer proteins and it may be that lipid mobility is, at least partially, under protein control.

Flip-flop can occur at significant rates when irregularities occur in the bilayer structure. In liposomes, flip-flop can be induced by triggering the formation of non-bilayer arrangements or by insertion of proteins that span the bilayer e.g. glycophorin (van Deenen 1981, Cullis and De Kruiyff 1979). Flip-flop in erythrocyte membranes has been shown to occur when the organisation of cytoskeletal proteins is disturbed (Haest et al 1978, Dressler et al 1984). Seigneuret and Devaux (1984) and Tilley et al (1986) have

shown that flip-flop of aminophospholipids from the external to the internal leaflet of erythrocyte membranes is an ATP-dependent process.

The Role of Phospholipids in the Activation of Factor X and Prothrombin

The prothrombinase complex, the tenase complex, and the extrinsic factor X activator complex are envisaged in the classical scheme of haemostasis as being comprised of an activating enzyme (Xa, IXa and VIIa respectively) a protein co-factor (Va, VIIIa and tissue factor apoprotein respectively), calcium ions, and a negatively charged phospholipid surface. Four of these proteins, factors VII, IX, X and prothrombin, known as the vitamin K-dependent proteins, contain two unusual amino acids. The first of these is γ -carboxyglutamic acid (see Fig 2.3) which occurs as a result of a postribosomal carboxylation of a number of glutamic acid residues of the proteins (Suttie and Jackson 1977, Davie et al 1979). It is thought that the γ-carboxyglutamic acid residues are essential for the calcium-dependent binding of vitamin K-dependent coagulation factors to negatively charged phospholipid surfaces. The binding parameters appear to be a function of pH, ionic strength, calcium concentration, type and mole fraction of acidic phospholipid in the membrane and the physical properties of the phospholipid preparation i.e. whether they are in the form of monolayers or vesicles. Nelsestuen et al (1978) compared the dissociation constants for protein-membrane complexes of various vitamin K-dependent factors with phospholipid vesicles of 20 mol% PS/80 mol% PC. The affinity

of the factors for this membrane decreased in the order Factor X-prothrombin-protein S-Factor IX-Factor VII-protein C and was not changed if the factors were activated. Nelsestuen and Broderius (1977) reported that PS- and PA-containing membranes have approximately the same affinity for coagulation factors while PG-containing membranes were less effective. This latter observation was confirmed by van Dieijen et al (1981) and is tested in the present study (see Chapter 8). Prothrombin contains 10 γ-carboxyglutamic acid residues (Magnusson et al 1975) and factor X contains 12-14 (DiScipio and Davie 1979). Resnick and Nelsestuen (1980) proposed that prothrombin-membrane binding can be explained by a model in which calcium forms ionic bridges between the two carboxyl groups of γ-carboxyglutamic acid in the protein and two negative charges of the phospholipid molecule in the membrane.

The second modified amino acid to be found in the vitamin K-dependent factors is β-hydroxyaspartic acid (see Fig 2.3) (Drakenberg et al 1983, McMullen et al 1983). The function of this amino acid has not yet been elucidated.

The protein co-factors of the prothrombin- and factor X-activating complexes also bind with high affinity to phospholipid membranes. Factors V and VIII do not contain γ-carboxyglutamic acid residues so an alternative to the calcium-dependent binding, described above, must be involved. The binding of factors V and Va requires acidic phospholipids (Bloom et al 1979, Pusey et al 1982). These groups compared the binding of factor V and Va. Pusey et al (1982) observed that the binding properties of factors V and Va were

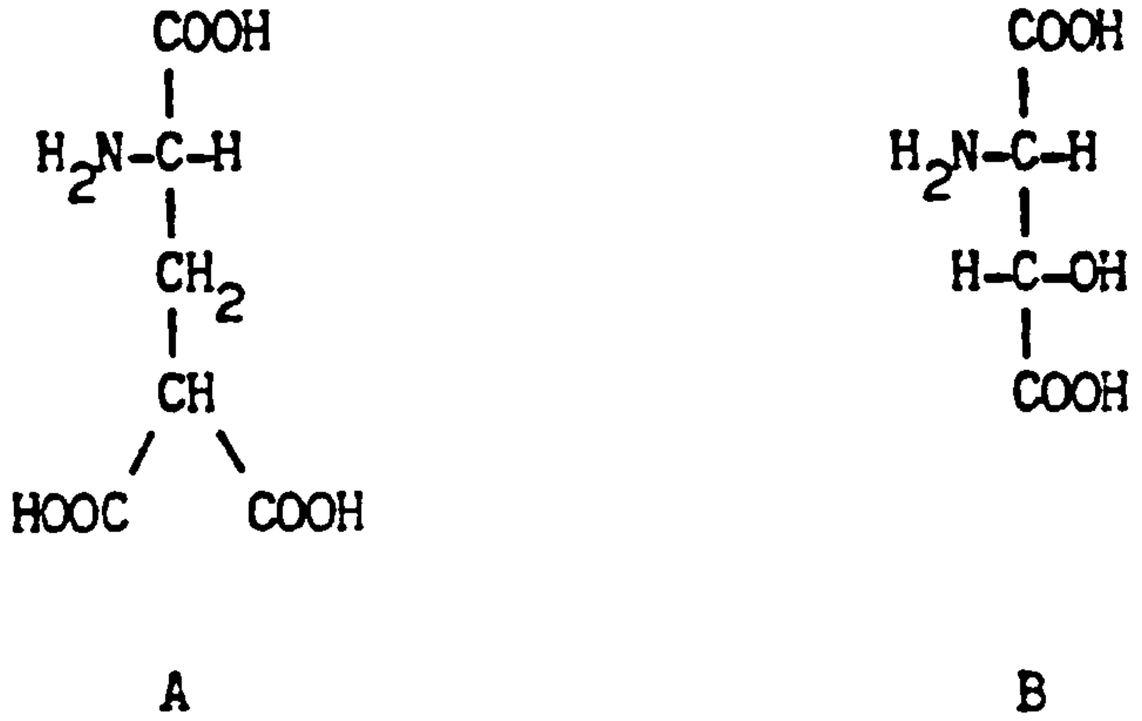


Fig 2.3 γ-carboxyglutamic acid (A) and β-hydroxyaspartic acid (B)

similar while Higgins and Mann (1983) were of the opinion that factor V binds with four to six-fold higher affinity than factor Va. Pusey et al (1982) and van de Waart et al (1983) suggested that factor Va-phospholipid interaction is primarily the result of ionic forces. Mann's group, however, held that ionic strength hardly affects the dissociation constant of the factor Va-phospholipid complex.

Isolated factor Va consists of two polypeptide chains of 95,000 daltons and 75,000 daltons which are noncovalently associated in the presence of calcium ions (Esmon 1979, Hibbard and Mann 1980). The heavy chain does not bind to phospholipid (van de Waart et al 1983, Higgins and Mann 1983) while the light chain, which is positively charged at physiological pH, has a high affinity for negatively charged membrane surfaces (van de Waart et al 1983, Higgins and Mann 1983, Pusey and Nelsestuen 1984). The binding parameters for the light chain are similar to those for the entire molecule, which suggests that the light chain of factor Va contains the peptide domain responsible for the binding of factor Va to membrane surfaces.

Andersson and Brown (1981) and Lajmanovich et al (1981) showed that when Factor VIII-von Willebrand complex was incubated with phospholipid vesicles containing PS, the coagulant activity (factor VIII:C) dissociated from the von Willebrand factor activity (factor VIIIR:WF) and bound to the phospholipid surface. Andersson et al (1981) suggested that factor VIII-phospholipid binding depends mainly on hydrophobic interactions. Factors V and VIII each have a

molecular weight, in the native state, of 330,000 daltons. Factor V is present in plasma at a concentration of 10 $\mu g \ cm^{-3}$ while factor VIII concentration is only 0.1 $\mu g \ cm^{-3}$.

It is now accepted that phospholipids function in the activation of vitamin K-dependent factors by providing a surface onto which the factors can bind. Jobin and Esnouf (1967) and Hemker et al (1967) proposed that the effect of phospholipids on prothrombin activation was the binding and localisation of the proteins at the phospholipid surface to facilitate their interaction. As a result of more recent studies, several models have been proposed to explain the role of phospholipids. The first of these is the "bound substrate model". In this model the phospholipid-bound enzyme-cofactor complex acts on phospholipid-bound substrate (Rosing et al 1980, Nesheim et al 1984). The overall substrate density at the phospholipid surface determines the rate of substrate activation. Since the bound substrate concentration greatly exceeds that in free solution, the interaction of substrate with phospholipid-bound enzyme is aided and results in an increased rate of factor activation.

The second model is the "free substrate model", in which the enzymatic unit acts on soluble substrate (Nelsestuen 1978). The affinity for soluble substrate is determined by the additional free energies of substrate binding to the individual components of the activating complex i.e. enzyme, protein co-factor, and phospholipid molecules. The presence of phospholipids in the enzymatic unit is

responsible for the increased affinity of the complete complex for its substrate.

Finally, model three is the "conformation model" in which the phospholipid-bound enzymatic unit has a conformational state which is different from that of the enzyme molecules in free solution. The conformational change induced by binding to phospholipid causes an increased affinity for soluble substrate molecules (Forman and Nemerson 1986, van Rijn et al 1986).

There is no reason to suppose that only one of these models is correct in all the activator complexes described. It is possible that different models work in different complexes or even within the same complex, depending on the presence or absence of protein co-factor, for example.

Involvement of Platelets in the Activation of Factor X and Prothrombin

Under normal conditions, platelets circulate in an inactive condition, until after 8 to 10 days when they are removed by the reticuloendothelial system. Normal adults produce new platelets at a rate of 1.5×10^{11} per day. These are flat, discoid cells with an average diameter of 2-3 microns and an average volume of approximately 8 cu microns (Karpatkin 1977, Stahl et al 1978). The plasma membrane of the platelet follows the fluid mosaic model described above but differs from other cells in the way in which it invaginates into the cell interior to form a system of channels,

(White 1972) known as the open canalicular system. This system serves to increase the surface area of cell in contact with the aqueous environment and to provide channels for products from the secretion granules to reach the plasma. A ring of microtubules preserves the disc shape. Electron microscopy reveals that the plasma membrane shows a typical lipid bilayer structure with an electron-dense glycocalyx extending outwards by as much as 25 nm. This is the carbohydrate rich domain of some 30 membrane glycoproteins exposed at the extracellular surface (Berndt and Phillips 1981). Glycoproteins (GP) Ib, IIb and IIIa span the bilayer and interact with cytoskeletal proteins on the cytoplasmic side (Fox 1985, Fox 1985).

The lipid bilayer of platelets contains 70% phospholipids by weight, the remainder being cholesterol and glycolipids. Marcus (1978) and Perret et al (1979) showed the presence of five major phospholipid classes i.e. PC (38%), PE (27%), SPH (19%), PS (10%) and PI (5%). The phospholipids are asymmetrically distributed in the bilayer (Zwaal 1978, Perret et al 1979, Zwaal et al 1977, Schick 1979) (see Chapter 4). The outer leaflet of the plasma membrane contains most of the SPH, while PS and PI are mainly confined to the inner leaflet. PE is distributed mostly on the inside while PC is more or less equally shared.

When a blood vessel is injured a plug of aggregated platelets rapidly forms when platelets contact sub-endothelial structures. At the same time the haemostatic mechanism leads to the formation of insoluble fibrin which reinforces the primary haemostatic plug to

form a stable thrombus. Thrombin, formed in the final stage of coagulation, is a potent platelet activator.

Platelets contain a number of proteins involved in the coagulation process. These are mostly located in storage granules and are released upon platelet activation. Platelets have three types of granules; dense, α, and lysosomal. The dense granules are few in number and appear as black round dots on electron micrographs. They are the site of storage of ATP-ADP and their deficiency results in a bleeding tendency that is proportional to the residual dense granule ADP (Akkerman et al 1983).

The granules contain a variety of proteins, including coagulation factors, proteins of the complement and fibrinolytic systems, and inhibitors of these systems. Some are specific for platelets e.g. platelet factor 4 and β-thromboglobulin. Platelet-derived growth factor (PDGF) is also released from granules and stimulates proliferation of vessel wall smooth muscle cells. This results in thickening of the vessel wall and reduction of anti-haemostatic properties and contributes to formation of an atherosclerotic plaque.

Among the non platelet-specific proteins of the α-granules are fibrinogen, fibronectin, factor V, plasminogen, high molecular weight-kininogen, von Willebrand factor, plasminogen activator-inhibitor, antithrombin III and protein S (Akkerman 1988).

Another group of α-granule proteins includes vascular permeability factor, chemotactic factor and thrombospondin. The latter is a protein of molecular weight 420,000 daltons that consists of three identical chains, each containing sites for binding to heparin, fibronectin, fibrinogen, plasminogen, type V collagen, calcium and sulphated glycolipids. The protein has been shown in megakaryocytes, aortic smooth muscle, fibroblasts, endothelial cells, monocytes and macrophages and its role in platelet aggregation is thought to be the reinforcement of the complex of fibrinogen with glycoproteins IIb-IIIa (GPIIb-IIIa) (Leung 1984, Lawler 1986).

Many other proteins bind to activated platelets. Fibrinogen, von Willebrand's factor and fibronectin bind to exposed GPIIb-IIIa (Plow and Ginsberg 1981, De Marco et al 1985). Activated platelets also bind HMWK, plasminogen, factors XIIIa, XIa, X, prothrombin and protein C though it is not known whether they require specific binding sites or bind to exposed negatively charged phospholipids (Greengard and Griffin 1985, Miles et al 1986, Greenberg and Shuman 1984, Harris and Esmon 1985).

Platelets contain at least 13 different lysosomal enzymes, whose importance is not yet established although it seems likely that they help to remove the haemostatic plug when damaged tissue is repaired (Akkerman 1988). The extent and rate of secretion among the three types of granule depends upon the type and concentration of the stimulus. Maximal secretion was found after a high dose of thrombin (5 NIHu cm^{-3}) which liberated all α- and dense-granule

contents after 2 minutes and approximately 50% of the lysosomal granules after 5 minutes. With lower doses of thrombin or weak activators such as adrenalin or ADP, α- and dense-granule secretion was about 50 to 70% and lysosomal secretion no more than 10% (Verhoeven et al 1984).

Two of the important procoagulant activities of the platelets, the provision of suitable phospholipid surfaces for the prothrombinase complex and the tenase complex, are made available when platelets are activated by specific stimuli. The negatively charged phospholipids are essential in a procoagulant phospholipid surface (Bangham 1961, Papahadjopoulos et al 1962) since they are able to bind coagulation factors via a Ca^{2+}-mediated interaction. In this way the K_m for prothrombin and factor X is decreased to values below the physiological concentration in plasma (Suttie and Jackson 1977).

Nesheim et al (1982) and Kane and Majerus (1982) showed that high-affinity binding of factor Xa to platelets requires the presence of factor Va. This is not the whole story, however, since des-(1-44)-factor Xa (which is a form of factor Xa lacking the N-terminal 44 amino acid residues containing γ-carboxyglutamic acid) has a more or less unchanged enzymatic activity towards prothrombin and unchanged affinity to factor Va in solution (Kane and Majerus 1982, Morita and Jackson 1986). It was demonstrated, nevertheless, that the modified factor Xa had a 100-fold lower affinity than factor Xa for platelet-associated factor Va. The rate of thrombin formation by des-(1-44)-factor Xa was shown to be 300-fold less than

that measured with factor Xa in a system of phospholipids and Va (Skogen et al 1984). In 1983, Dahlback et al partially purified a lupus antibody that reacted against negatively charged phospholipids and demonstrated that it blocked prothrombinase activity in the presence of platelets as well as phospholipids. These observations support the idea of an essential role for the negatively charged phospholipids for the activation of prothrombin at the platelet surface. Rosing et al (1985) and Bevers et al (1982) used phospholipases to destroy the prothrombinase and factor X-activating activity of nonactivated platelets. They found that attacking membrane phospholipids other than PS did not produce this result. Proteolytic treatment of unstimulated platelets did not affect the ability to stimulate these two platelet activities. These data indicate that the binding sites for both complexes of factors Xa-Va and factors IXa-VIIIa are not essentially different from each other and consist of negatively charged phospholipids at the outer surface of the platelet.

There are severe limitations concerning the accuracy with which the amount and composition of phospholipids at the platelet outer surface can be detected. One problem is that platelet activation will lead to platelet aggregation and the amount of outer surface available for probes will be reduced. Another problem is that the platelet release reaction involves a secretory event during which the granule membranes fuse with the plasma membrane, increasing or altering the amount of phospholipids at the surface membrane. The use of chemical probes and highly purified phospholipases in this work has been described by Schick (1978) and

Bevers et al (1983) and is considered in Chapter 4, below. The latter group believed that the alterations in phospholipid composition of the outer membrane leaflet of platelets, activated by thrombin or collagen alone, can be attributed to the secretory event which involves fusion of granule membranes with plasma membrane. The progressive loss of phospholipid asymmetry in platelets activated by collagen plus thrombin, diamide, or Ca ionophore A23187 presumably reflects an abrupt increase in transbilayer movement of phospholipids as a result of the activation procedure.

The mechanism, or mechanisms, by which the negatively-charged phospholipids can move from the inner to the outer leaflet of the plasma membrane upon platelet activation are not completely understood. One suggestion for such a mechanism is that non-bilayer arrangements may be triggered which can facilitate the flip-flop translocation of phospholipids. In artificial bilayers, flip-flop can be induced by creating different physical properties between outer and inner leaflets, by insertion of bilayer-spanning proteins or by causing non-bilayer arrangements in the membrane (van Deenen 1981, Cullis and de Kruiyff 1979). In red cell membranes it has been shown that increased flip-flop occurs when the organisation of cytoskeletal proteins is disturbed (Haest et al 1978, Franck et al 1985).

Chap and Douste-Blazy (1974) treated platelets with a phospholipase C from <u>clostridium welchii</u>, which resulted in release and aggregation and eventually to lysis of the cells. Comfurius et al (1983) used a combination of phospholipases, at sublytic

concentration and showed that substantial amounts of PS appeared at the external surface. The exposure of PS was accompanied by an enchanced thrombin formation by the prothrombinase complex. Phosphatidic acid is formed during these treatments, although at 2-4% of total phospholipid, too little to account for the observed prothrombinase activity. This is the same quantity of PA that is formed in activated platelets during the triggering of the phosphatidyl inositol cycle (Lapetina and Cuatrecasas 1979, Bell and Majerus 1970). In the initial steps of the PI cycle, polyphosphoinositides are converted to diacyl-glycerol by an endogenous phospholipase C, followed by a phosphorylation catalysed by diacyl glycerol kinase to form PA (Rittenhouse 1983). Enough diacylglycerol and PA may be produced to induce the formation of non bilayer arrangements and thereby to form a locus for flip-flop of phospholipids, PS in particular, to the outer leaflet (a further discussion of the PI cycle is to be found in Chapter 8.10).

It has been demonstrated that, in the red blood cell, phospholipid asymmetry depends upon cytoskeletal proteins (Haest et al 1978, Dressler et al 1984, Franck et al 1985). SH-oxidising agents such as diamide or tetrathionate were employed in these studies to induce changes in the structural organisation of the cytoskeletal proteins, and phospholipids were found to move from inner to outer leaflet following cross-linking of spectrin. Davies and Palek (1982) treated platelets with diamide and reported polymerisation of cytoskeletal proteins, notably filamin, myosin and actin. This process was accompanied by movement of aminophospholipids from inner to outer leaflet (Bevers et al 1983).

Treatment of platelets with the Ca ionophore A23187 in the presence of calcium chloride caused similar polymerisation but also hydrolysis of actin-binding proteins filamin and talin under control of an endogenous calcium-dependent protease (Truglia and Stracher 1981, McGowan et al 1983). The endogenous calcium-dependent protease can also be stimulated when platelets are activated by physiological activators. Proteolysis decreased in the order A23187 > collagen + thrombin > collagen > thrombin = ADP. The same order was found for the ability of these activators to induce exposure of anionic phospholipids on the outer leaflet of the platelet plasma membrane, and to stimulate platelet procoagulant activity (White 1980, Fox et al 1985, Comfurius et al 1985). Further proof of the involvement of cytoskeletal proteins in maintenance of phospholipid asymmetry came from observations made by Solum and Olsen (1985) and Verhallen et al (1986) who examined the actions of the local anaesthetics dibucaine and tetracaine on platelets. Extensive degradation of cytoskeletal proteins resulted and an increased ability of treated platelets to stimulate prothrombinase activity.

Yet another possibility must be considered. Observations with red blood cells suggest that movement of aminophospholipids is ATP-dependent (Seigneuret and Devaux 1984, Tilley et al 1986). Since myosin is the major membrane component with which ATPase activity is associated (Peleg et al 1984), it may be that cleavage of membrane-bound myosin may inactivate myosin-ATP ase, and deprive the membrane of energy, with the result that membrane asymmetry can not be maintained.

2.2 SOME ANTICOAGULANT MECHANISMS

The Effect of Dicoumarol on the Biosynthesis of Clotting Factors

Almost seventy years ago, it was observed by the farming community of Ontario and North Dakota, that a serious bleeding disease of cattle was caused by consumption of spoiled sweet clover. The observation was investigated (Schofield 1922, Roderick 1929) and the disease shown to be associated with a prothrombin deficiency. The causal agent from sweet clover was identified as 3,3-methyl-bis-4-(hydroxycoumarin), called dicoumarol, presumably formed by the action of microorganisms on coumarin (Campbell and Link 1941). It was soon pressed into service as an anticoagulant (Bingham et al 1941, Butt et al 1941).

Previously, the work of Dam had shown that the absence of a fat-soluble factor caused a defect in blood coagulation. This factor was named "Koagulations" vitamin (vitamin K). The active compound was phylloquinone (vitamin K_1) and its characterisation led to Dam and Doisy sharing a Nobel prize in 1943 (Dam and Doisy 1964). The effect of dicoumarol on the biosynthesis of clotting factors II, VII, IX, X, protein C and protein S is similar to a deficiency of vitamin K i.e. synthesis of biologically active factors is reduced but is rapidly restored by administration of the vitamin.

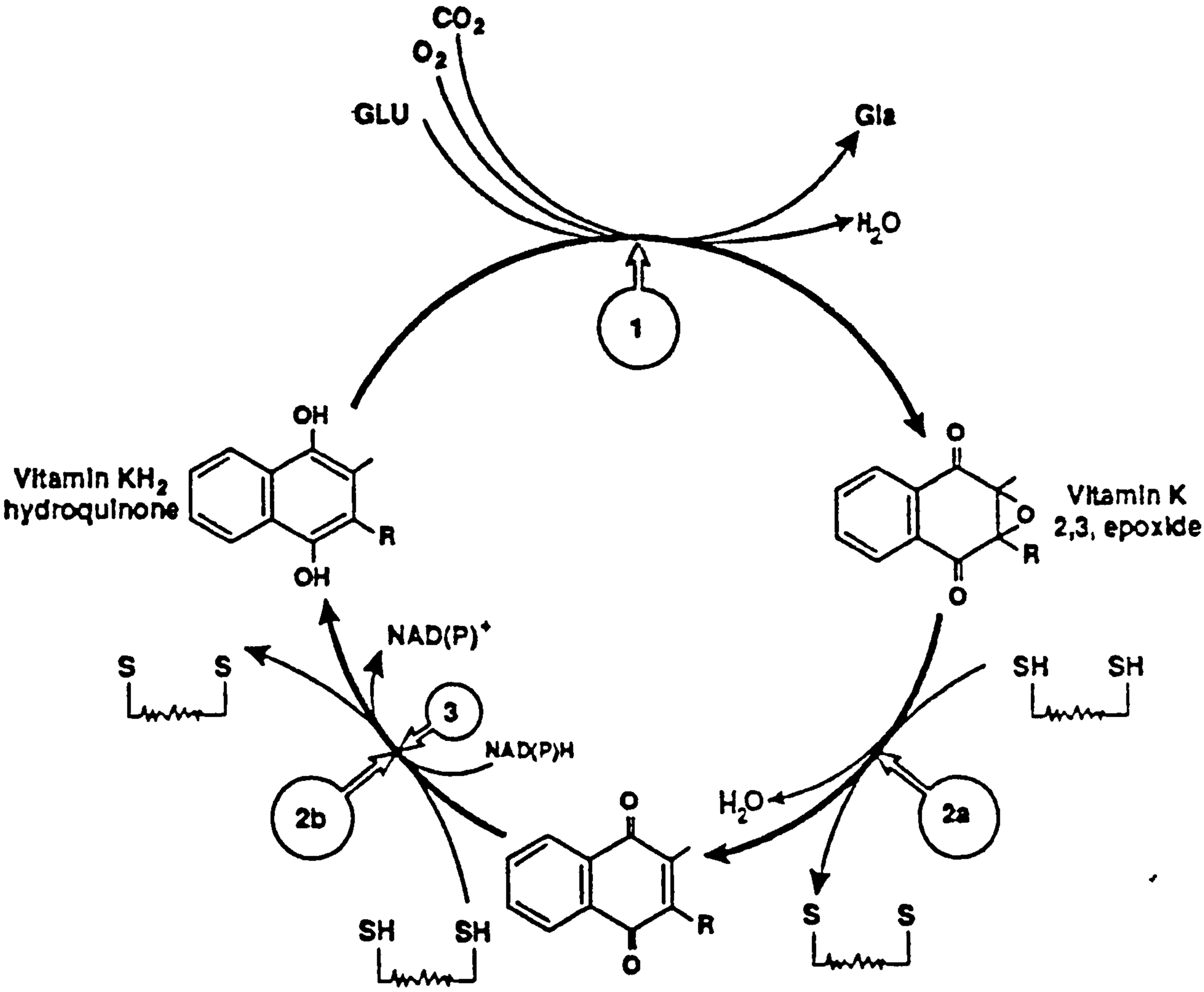


Fig 2.4 Vitamin K metabolism in rat liver microsomes.

(modified from Stenflo et al. 1974)

Stenflo et al (1974) and Nelsestuen et al (1974) showed that prothrombin contained a number of residues of a previously unidentified amino-acid, γ-carboxyglutamic acid (Gla) (See Fig 2.3). Vitamin K metabolism in hepatic microsomes is illustrated in Fig 2.4. In addition to the carboxylase/epoxidase system (labelled 1 in the figure), liver microsomes contain a dithiol-linked vitamin K epoxide reductase (labelled 2a) and a dithiol-linked vitamin K quinone reductase (2b). These two dithiol-linked reductase activities are strongly inhibited by dicoumarol. The NADPH-linked quinone reductase activity (labelled 3 in Fig 2.4) is catalysed by at least two different enzymes. These are less sensitive to dicoumarol inhibition than, for example, dithiothreitol-dependent vitamin K quinone reductase (Fasco and Principe 1982) and provide a pathway for vitamin K quinone reduction in anticoagulated patients (Wallin et al 1986). The presence of this pathway explains the ability of administered vitamin K to counter the effect of dicoumarol. Four of the vitamin K-dependent clotting factors, i.e. II, VII, IX and X have been described earlier. Two further vitamin K-dependent proteins (protein C and protein S) will now be considered.

The Roles of Protein C and Protein S

Protein C has a molecular weight of 65,000 daltons, is present in plasma at a concentration of 4 $\mu g\ cm^{-3}$, and is composed of two disulphide linked chains of 17,000 and 28,000 daltons. Unlike the vitamin K-dependent proteins described above, protein C zymogen activation leads to the generation of an anticoagulant enzyme. The protein can be cleaved by thrombin in the amino-terminal region of

the heavy chain. The release of a 14 amino acid activation peptide results in the formation of activated protein C, which is able to inactivate both factors Va and VIIIa (Kisiel et al 1977, Vehar and Davie 1980).

Thrombin can convert protein C to activated protein C (Kisiel 1979). In vitro this reaction is quite slow. Esmon et al (1982), however, isolated a protein of approximately 74,000 daltons molecular weight, from rabbit lungs, which has a high affinity for the enzyme. This protein, called thrombomodulin, forms a 1:1 stoichiometric complex of enzyme and cofactor that is able to activate protein C rapidly in the presence of Ca^{2+}. Thrombin attached to thrombomodulin can be neutralised by antithrombin at the same rate as the free enzyme. The ability of the bound enzyme to clot fibrinogen, activate factor V or trigger platelet activation is considerably diminished. Thus, the endothelial cell receptor, thrombomodulin, has the ability to accelerate the rate of thrombin-dependent protein C activation, while allowing inactivation of bound thrombin by antithrombin and inhibiting the procoagulant activities of the enzyme.

Once activated, protein C functions as an inhibitor of factors V and VIII. One site of action is located at the surface of the platelet where factor Va acts as a receptor for factor Xa i.e. the prothrombinase complex. Factor Va appears to be particularly sensitive to destruction by activated protein C especially in vivo when concentrations of the enzyme are very low (Walker et al 1979, Dahlback and Stenflo 1980).

Another site of action of activated protein C is at that point where factor VIIIa regulates the interaction between factor IXa and factor X i.e. the tenase complex (Vehar and Davie 1980).

Protein S acts as a cofactor to facilitate the action of activated protein C on factors Va and VIIIa (Suzuki et al 1984). It has a molecular weight of 69,000 daltons (DiScipio and Davie 1979) and is present in plasma at a concentration of 25 $\mu g\ cm^{-3}$. Thrombin inactivates the anticoagulant activity of protein S (Suzuki et al 1983). In plasma about 60% of protein S is bound to complement factor C4b. In this form it does not appear to be an active cofactor for activated protein C (Dahlback 1986).

The Action of Heparin

The action of heparin appears to depend upon binding to antithrombin III (AT-III), a protein of 58,000 daltons molecular weight, which is present in plasma at a concentration of 150 $\mu g\ cm^{-3}$ (Rosenberg et al 1984). AT-III neutralizes the activity of thrombin and the other serine proteases, except factor VII (Hemker 1987). Heparin acts by facilitating the interaction of the protease and the antiprotease. Once the reaction is complete, heparin leaves the complex. Griffin (1982, 1983) showed that random order kinetics apply to heparin catalysed AT-III protease interaction. This implies that heparin acts as a template on which the proteins meet. Other authors feel that an ordered bireactant model would be more appropriate (Jordan et al 1980, Fletcher et al 1985). According to

this model, heparin binds first to AT-III to produce an allosteric change that increases the efficiency of the inhibitor. Jordan et al (1980) showed that, at optimal concentrations, heparin accelerates the rates of inhibition of thrombin and factor Xa by AT-III by at least 1000-fold. Heparin is a sulphated uronic acid-containing polysaccharide with potent anticoagulant activity. The repeating subunits of the polysaccharide are the disaccharides D-glucuronic acid-D-glucosamine and L-iduronic acid-D-glucosamine, the first pair linked by an α-D (1 → 4) bond and the latter by an α-L (1 → 4) bond (Kiss 1974). Multiple sulphate groups are linked to both glucosamine and iduronic acid residues. Heparin from different species may vary in the proportions of the different disaccharide subunits and in the numbers of N and O sulphate residues (Kiss 1974). Despite these differences heparins all show anticoagulant properties, which appear to be related to the stereochemistry of the molecule (Rosenberg et al 1978).

The region of the molecule that is required for binding AT-III has been identified by affinity chromatography of depolymerised heparin on columns with bound AT-III (Lindahl et al 1979, Radoff and Danishefsky 1984). The AT-III binding region is contained in a pentosaccharide sequence in which the central glucosamine has a unique O-sulphate group. Four of the seven sulphate groups in this pentasaccharide sequence are critical for binding AT-III with high affinity (Lindahl et al 1979, Atha et al 1984).

The polysaccharide chains in commercial heparins are heterogeneous both in composition of the repeat disaccharides and

with respect to chain length (Casu 1985, Hook et al 1984). The average molecular weight of heparin is 12,000 daltons, but species ranging from 6,000 to 25,000 (i.e. chain lengths varying from 18-70 monosaccharide units) are present in commercial heparin preparations.

Hemker (1987) described a series of experiments in which it was shown that AT-III-heparin had a pronounced effect on factor IXa. He believed that heparin action on free factor IXa is a secondary effect while inhibition of thrombin is the major one. He concludes that heparin acts on thrombin and on free factor IXa, incidentally, but not on factor Xa in the prothrombinase complex, nor on factor IXa in the tenase complex. For this reason he sees no use for measurement of anti-Xa but prefers the use of the APTT test for routine use in measurement of heparin activity.

CHAPTER 3 TISSUE THROMBOPLASTIN

HISTORICAL REVIEW - THE BIOLOGY OF TISSUE THROMBOPLASTIN:

In 1819, Thackrah reported that blood clotted more rapidly if it flowed over an exposed tissue than if it were carefully collected in a container. Wooldridge (1893) also noted the potent coagulant activity of tissue extracts and thought they contained a substance which converted prothrombin to thrombin. He called this substance thrombokinase. He went further by injecting tissue extracts, of varied origins, into animals and observed no lethal effect if the preparations were first extracted with alcohol-ether. Morawitz (1904) proposed his "Classical" theory of blood coagulation. He believed that thrombokinase, in the presence of calcium ions, converted prothrombin to thrombin. The term thromboplastin was first coined by Nolf (1908). He had a similar theory to that of Morawitz and considered tissue extract to be a "coagulant of the third order".

Howell (1912) believed thromboplastin to be a complex of protein and lipid, which had the ability to remove an inhibitor from prothrombin, allowing it to react with calcium ions to form thrombin. Howell, even in 1952, still used the term thrombokinase for the protein of the tissue procoagulant. Thromboplastin was the term he applied to the lipid moiety. Mills (1921) confirmed the observations made by Wooldridge in his animal experiments. He prepared a thromboplastin from bovine lung and treated it with alcohol-ether. He was able to recombine his alcohol-ether soluble

fraction with the alcohol-ether insoluble fraction and recover some activity.

This experiment set the fashion for years to come and a variety of workers since then have improved on this basic experiment of purifying a lipid component and a non-lipid component and recombining the two in an attempt to restore activity. Chargaff et al (1944) for example, purified a particulate fraction of lung by homogenization, precipitation and differential centrifugation. This material contained lipid and proteins. By prolonged extraction with organic solvents, a lipid-free protein fraction was obtained, which did not possess coagulant acitivity. It was concluded that both fractions were necessary for full activity. Studer (1946) extracted tissues with alcohol-ether. He obtained two fractions - insoluble protein and soluble lipids, neither fraction showing activity. When recombined and the solvent evaporated, activity was restored. Chargaff (1948) meanwhile was still concerned with attempts to reassociate the lipid and protein moieties. Lung thromboplastin particles were incubated in 1 mol dm^{-3} NaCl, 5 mol dm^{-3} guanidine HCl or 0.012 mol dm^{-3} sodium deoxycholate and then centrifuged at 31,000 x g. The pellets from the first two treatments contained all the activity, whereas the supernatant from the deoxycholate treatment was active, i.e., the activity was solubilized by the deoxycholate and did not sediment in the centrifuge. On removal of the deoxycholate by dialysis, protein and lipid reassociated with restoration of coagulant activity.

Kuhn and Klesse (1957) were the first to substitute various single natural and synthetic phospholipids for the crude lipid fractions that had been used previously. Deutsch et al (1964) extracted brain particles with pyridine and showed that the protein residue required the pyridine-soluble material for activity. The nature of the pyridine-soluble fraction was not investigated. Williams (1964,1966) working with human lung, placenta and brain, and Clarke and O'Meara (1966) also working with placenta, showed that the thromboplastic properties of tissue reside in the microsomal fraction.

Nemerson (1968) showed that phospholipids were definitely required for tissue factor activity and that it was the extrinsic system which was involved. He observed that while a crude mixture of brain lipids augmented the activity of tissue factor, PE was most effective in restoring activity to the protein residue after lipid extractions. PC was also effective, though less so.

Extending Chargaff's work, Hvatum and Prydz (1966) solubilized brain particles in sodium deoxycholate and demonstrated by gel filtration, on Sephadex G200, that the activity was associated with a protein of molecular weight about 50,000 daltons. If deoxycholate was omitted or removed prior to gel filtration, large aggregates were formed and the activity emerged in the void volume.

Nemerson (1969) used deoxycholate solubilization in purifying proteins from bovine lung and brain. He removed lipid by heptane/butanol extraction from acetone dried powders and

solubilized the proteins with deoxycholate. The proteins precipitated by ammonium sulphate at 30 - 60% saturation, contained less than 1% of phospholipid and were not active as thromboplastin. However, when recombined with phospholipids, and deoxycholate removed, activity increased by 500 - 1,000 times. PE was the most active, followed by PC. PS was inert. Mixed phospholipids were more active than PE alone. Nemerson and Pitlick (1970) further purified the protein component of bovine thromboplastin and found it to comprise two major species of molecular weight 330,000 and 220,000 daltons, the smaller having the higher specific activity. Pitlick et al (1971) reported peptidase activity associated with the thromboplastin protein. Several other groups, e.g., Liu and McCoy (1975) and Gonmori and Takeda (1976) performed similar purifications of thromboplastin protein from various sources and observed activity associated with proteins of molecular weights ranging from 56,000 daltons for bovine brain to 250,000 daltons for human placenta.

This confusing picture may be due to the hydrophobic nature and therefore insolubility in aqueous systems of the apoprotein. If decxycholate treatment is insufficient there will be formation of large aggregates of protein with apparently very high molecular weights. These aggregates may even appear as homogeneous bands in polyacrylamide gels.

Bjorklid et al (1973) used ultracentrifugation and solubilization with deoxycholate, combined with gel filtration on G200 and G100 columns and finally polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE) to obtain a protein of molecular

weight 53,000 daltons from human brain. This protein showed no peptidase activity. Bjorklid et al (1987) have recently shown, however, that this procedure yields material which is heterogeneous. The present author obtained a value of 55,000 daltons for the molecular weight of human brain tissue factor, also using sodium deoxycholate to solubilise the protein (Stevenson 1978).

Another advance in methodology came with the use of the nonionic detergent Triton X-100 to replace the bile salts. Bach et al (1981) described the use of Triton X-100 combined with lectin-affinity chromatography and preparative SDS-PAGE to purify bovine brain tissue factor to homogeneity. This technique yielded a polypeptide with an apparent molecular weight of 40,000 to 43,000 daltons depending on the gel system employed (Bach et al 1986). This material was used to produce polyclonal antibodies which were used in an immunoaffinity column to purify larger yields of tissue factor. Carson et al (1985) produced two monoclonal antibodies against bovine tissue factor which were also employed in inmmunoaffinity columns, improving the efficiency of the system over the polyclonal method. The homogeneity of the bovine brain tissue factor was assessed by proteolytic digestion. Tryptic digestion of a Triton X-100 solubilised preparation removed a small peptide, decreasing the molecular weight from 43,000 to 40,000 daltons, but without altering procoagulant activity of the protein. The protein and the procoagulant activity comigrated on SDS gels before and after proteolysis, establishing homogeneity (Bach et al 1981, Bach 1988).

More recently, Broze et al (1985) and Guha et al (1986) have reported the isolation of homogeneous human brain tissue factor by affinity chromatography using human factor VII coupled to agarose. The procoagulant activity in a Triton X-100 extract bound to the column. This binding was calcium-dependent and a highly purified protein was eluted from the column after chelation of the calcium. Carson et al (1985) have also produced a monoclonal antibody and used it to purify tissue factor from human brain and placenta. The proteins prepared in this way are identical as judged by amino-acid composition, N-terminal sequence, and procoagulant activity.

Since the tissue factor protein alone was devoid of any activity, it was essential to recombine it with phospholipids, remove the detergent and look for procoagulant activity. This very cumbersome procedure has to be performed at every stage in the purification process. A ratio of 1:80 for human apoprotein:phospholipid was shown to be optimal by Bjorklid and Storm (1977). While there is not complete agreement on the nature of the phospholipids required for optimal reconstitution of procoagulant activity, it is clear, however, that mixtures of phospholipids containing negatively charged molecules, for example PS or PG are most effective (Wijngaards et al 1977, Bjorklid and Storm 1977). A requirement for unsaturation has also been demonstrated (Nemerson 1968, Wijngaards et al 1977) implying that a liquid crystalline phase in the lipid bilayer is essential for proper expression of procoagulant activity.

Some of the earlier work, in which individual phospholipid classes were found to confer activity, may not have employed sufficiently pure lipids to justify confidence in the results.

The problem of the anatomical location of tissue factor has been addressed by a number of workers over a great many years. Bernheim (1910), for example, found that homogenates of aorta significantly shortened the clotting time of plasma. Campbell (1954) extended this work by dissecting bovine aorta into its three layers. He found the adventitia to be most active, followed by the media. The intima he found to have little or no activity. Kirk (1962) and Astrup and Buluk (1963) performed similar experiments and found the opposite to be the case. Astrup (1965) found that brain, lung, thyroid gland, adipose tissue and placenta were rich sources of tissue thromboplastin while none could be detected in liver.

Zeldis et al (1972) localised tissue factor with peroxidase conjugated antibodies and demonstrated the presence of the protein in the plasma membrane of many cell types, e.g., in intimal tissue of human vessels, in kidney tubular cells (in medulla and cortex), in hepatocytes, in the sarcolemma of myocardium, in coronary arteries and plasma membranes of alveolar cells. Zacharski and McIntyre (1973) corroborated these findings and showed the presence of tissue factor in cultured human fibroblasts.

Bjorklid et al (1977) employed goat and rabbit-anti human brain thromboplastin antibodies to examine the distribution of thromboplastin in human brain. Dissection of thirty four distinct

areas of brain provided the test material. The findings were that thromboplastin was located in phylogenetically older regions of the brain, implying that it represents a primitive or fundamental feature in the evolutionary sense, and there was no relationship between the distribution pattern of the protein and vascularisation.

It has been shown that a number of cells and tissues are able to synthesize tissue factor. Maynard et al (1977) showed this for fibroblasts and smooth muscle cells, while Dalaker et al (1983) and Dalaker and Prydz (1983) observed the same phenomenon in glioma cells and trophoblasts.

A second group of cells, which do not normally synthesize tissue factor, can be induced to do so by a variety of agents generated in pathological states. Prydz et al (1979) showed that immune complexes are able to induce tissue factor synthesis. Rivers et al (1975) showed that endotoxins were efficient inducers, while lymphokines have been implicated by Greczy and Hopper (1981). Other agents named as inducers include lectins, e.g., concanavalin A, wheat germ agglutinin as well as complement factor C3b, tumour promoters, thrombin and the calcium ionophore A 23187. All these observations were made in monocytes, macrophages and endothelial cells. Galdal et al (1984) showed that endothelial cells treated with histamine and epinephrine also synthesized thromboplastin. Prydz and Pettersen (1988) suggested that endothelial cells, like monocytes and macrophages, respond non-specifically to perturbations by synthesizing tissue factor. They point out that tissue factor activity peaks after 4 - 12 hours with most inducing agents and

decays within a similar period, even when the inducer remains present. Another aspect of the process is the effect of cellular co-operation as demonstrated by Lyberg et al (1983) for mixtures of endothelial cells and lymphocytes and by Johnson et al (1983) for endothelial cells and platelets.

Prydz and Pettersen (1988) hypothesise that tissue factor is synthesised as any other membrane protein. It contains a signal sequence which directs it into the endoplasmic reticulum and a hydrophobic domain which provides anchorage in intracellular and plasma membrane. The final assembled molecule is located in the plasma membrane with its factor VII binding end protruding into the extracellular medium. After addition of an inducing agent, a delay of about 60 minutes occurs before thromboplastic activity is detectable on intact cells. The fate of the formed tissue factor is still unknown but the transient nature of the response to induction suggests that mechanisms must exist for its rapid decay, even in the continuing presence of inducer.

Hetland et al (1985) have shown that tissue factor from monocytes is shed in vesicles from the plasma membrane as well as undergoing an apparent intracellular degradation. Bona et al (1987) examined the process of shedding in a monocyte-like cell line HL-60. They showed that tissue factor was indeed shed in membrane vesicles and that this process could be partially inhibited by tunicamycin and tunicamycin homologues, presumably by their inhibition of glycosylation which, in turn, will regulate intracellular transport of the molecule.

The next step in the structural characterisation of human tissue factor was the isolation of cDNA clones coding for the protein. Spicer et al (1987) have published the cDNA sequence of human tissue factor. Confirmation of this coding sequence, as well as portions of flanking cDNA has been reported by Morrisey et al (1987) and Scarpati et al (1987). The consensus of opinion is that the tissue factor molecule contains three domains. Firstly, there is a short cytoplasmic domain of 21 residues, including one cysteine that is probably not disulphide linked in the cell, although following cellular disruption it might well participate in disulphide linked dimer formation. Secondly, there exists a transmembrane region of 23 residues of a highly hydrophobic nature that probably forms an α-helical structure within the membrane and acts as an anchor (Spicer et al 1987). Finally, residues 1 to 219 form an extracellular domain which is hydrophilic. Four N-linked carbohydrate attachment sites occur in the sequence. Three of these are contained in the extracellular domain and two of these have been confirmed by protein sequencing as sites of post-translational modification (Spicer et al 1987, Morrisey et al 1987, Scarpati et al 1987). The fourth of these sites is within the cytoplasmic domain. The attachment of carbohydrate to the extracellular domain is evidence of the location of this portion of the molecule on the cell surface. Removal of carbohydrate from human tissue factor by endoglycosidase F caused no effect on its procoagulant activity, suggesting that this post-transitional modification must be required for some purpose other than expression of biological activity (Bach 1988).

The location of the human gene was mapped by Carson et al (1985). This was achieved by a mouse-human somatic cell hybridisation experiment which took advantage of the observation that mouse tissue factor does not clot human or bovine plasma (Kadish et al 1983, Lyberg et al 1982, Janson et al 1984). The gene was mapped to the short arm of human chromosome 1 (1pter-1p21). This was taken as evidence for a single tissue factor gene in the human. This result was confirmed by Scarpati et al (1987). Spicer et al (1987) examined the primary sequence of human tissue factor for homology against 4,668 sequences in the data base of the National Biomedical Research Foundation as well as several procoagulant and anticoagulant proteins not yet in the data base. No significant homologies were observed, suggesting that the protein has its origin, in phylogenetic terms, separate from that of the other coagulation factors. This may be consistent with its unique role as the initiator of a proteolytic cascade. This role is examined in Chapter 2.

Drake et al (1989) returned to the problem of the anatomical localisation of tissue factor. For this work they employed three murine monoclonal antibodies that reacted with human tissue factor. Two of these antibodies recognised functional epitopes on the protein, inhibiting tissue factor initiated coagulation and inhibiting specific binding to factor VII respectively. The third antibody recognised a distinct nonfunctional epitope and was used to ensure that the protein would still be revealed in tissues that may have bound factor VII in vivo.

This group showed that tissue factor in blood vessels was localised in the adventitia, being undetectable in endothelium and present in the media, although with large variability. Most muscular arteries and larger arterioles had activity in the adventitia, less so in kidney and gut submucosa but greater in spleen and superficial cerebral vessels. Capillaries and postcapillary venules did not react at any site. Adventitial cells of small to medium sized veins reacted similarly to those of arteries of comparable size and location. Unstimulated peripheral blood leukocytes were uniformly negative. Incubation of cells with endotoxin induced tissue factor in monocytes but not in neutrophils or lymphocytes. Eosinophils, basophils, erythrocytes and platelets also were negative.

Tissue factor was found in epidermis, bowel and respiratory mucosa, cerebral cortex, myocardium and renal glomeruli as well as in cells composing the fibrous capsules of liver, spleen, kidney and adrenals. Expression of tissue factor by connective tissue fibroblasts was not common, occurring most prominently in the adventitia of blood vessels and in the subepithelial fibroblastic sheath of bowel mucosa. Fibroblasts elsewhere were generally negative. These workers believe that tissue factor is "anatomically sequestered" from the blood in the normal state by this distribution pattern, which provides an envelope surrounding blood vessels in adventitia, encasing organs in fibrous capsules and showing a strong presence in epidermis and mucosal epithelium, thus providing a continuous haemostatic barrier with the external environment. They

suggest, also, that although much of the distribution of tissue factor in normal tissue can be understood in terms of its procoagulant function, its selective expression at sites not of obvious importance for haemostasis raises the question of whether the protein has additional functions.

Further investigation of the localisation of tissue factor and tissue factor mRNA in normal vessels and in human atherosclerotic plaques, obtained from carotid endarterectomy, was undertaken by Wilcox et al (1989). They found extensive mRNA hybridisation in several regions of the atherosclerotic plaque. Positive cells were found throughout the fibrous cap, base and shoulder region of the plaque, as well as in the necrotic core adjacent to the cholesterol clefts. The normal media underlying the endarterectomy specimens did not contain mRNA-positive cells or tissue factor protein. The necrotic cores of the plaques were characterised by the presence of extensive tissue factor deposition. Additional tissue factor protein staining was found in the macrophage-rich foam cell regions of many plaques. Such foam cell regions often lay underneath the fibrous cap and adjacent to the necrotic cores. No tissue factor mRNA or protein was detected in either the endothelium lining the vascular surface or the small vessels within the plaques. All sixteen endarterectomy specimens examined showed positive tissue factor staining in some region of the plaque.

This group believe that tissue factor protein found in the necrotic core may be shed from the surfaces of synthesizing cells and trapped in the surrounding matrix or, alternatively, that it may

originate from cells that have died and left tissue factor-rich membranes behind. The production of tissue factor by macrophages has been demonstrated by several workers e.g. Tipping et al (1988), Levy et al (1981). What could not be demonstrated in Wilcox's study was whether the tissue factor was manufactured by macrophages or whether it was ingested from necrotic core debris.

Examination of the normal vessels i.e. human saphenous vein and internal mammary artery samples showed that endothelial cells contained no tissue factor mRNA or protein. Some saphenous vein tunica media cells contained tissue factor mRNA but the immunohistochemical reaction was very weak. The strongest labelling was seen over the adventitia where intense staining of tissue factor protein and mRNA hybridization were found. Adventitial fibroblasts contained much more tissue factor protein than did the medial cells.

It has been suggested that plaque rupture is the event that precipitates clot formation (Forrester et al 1987, Friedman and van den Bovenkamp 1966). Plaque rupture that exposes the necrotic core region to the lumen has been shown by several groups to underlie thrombi in coronary and cerebral arteries (Falk 1983, Constantinides 1967). Tissue factor, therefore, is available in atherosclerotic plaque and may be the initiator of the thrombosis associated with plaque rupture.

THROMBOPLASTIN AS A LABORATORY REAGENT

As discussed in Chapter 2, the haemostatic mechanism may be dramatically retarded by the action of dicoumarol. The laboratory control of the dosage of this anticoagulant depends upon the use of the prothrombin time test, described originally by Quick (1935). A number of modifications of this test have been developed, e.g., Owren and Aas (1951) introduced the "P and P" test (prothrombin and proconvertin), in which the test sample was diluted and mixed with a source of factor V and fibrinogen prior to testing. It was claimed that the addition of factor V allowed the plasma to be tested several hours after collection. When it became known that the coumarin drugs reduced the active level of factor IX as well as factors II, VII and X, Owren (1959) devised the Thrombotest reagent as a means of monitoring change in all four factors. In the Thrombotest, the intrinsic pathway is accelerated by a potent partial thromboplastin, while the extrinsic pathway is initiated only slowly by a low-activity tissue thromboplastin. The reagent also contains bovine plasma stripped of factors II, VII, IX and X. This reagent is available commercially in lyophilised form and is reconstituted with a solution of $CaCl_2$.

This proliferation of methods caused much confusion for several years - confusion, both in techniques and expression of results. The test which has been used in the majority of centres throughout the world is Quick's one stage prothrombin time, in which tissue extract (thromboplastin) is used to accelerate the clotting of recalcified plasma. The British Committee for Standards in Haematology approved a reference method for the one-stage

prothrombin time test on human plasma which laid down a well controlled technique (Poller 1970). Given such a technique, and assuming the operator to be reasonably skilful, two areas remained where standardisation was still required, i.e., expression of results and the tissue thromboplastin reagent. The use of the "prothrombin index", which is the ratio of prothrombin time of normal plasma to the prothrombin time of patients plasma, expressed as a percentage, was widespread as was the use of the term "prothrombin activity". The latter required construction of a dilution curve of normal plasma, employing saline or $BaSO_4$ adsorbed plasma as diluent, from which the patients prothrombin activity could be read. To further confuse matters, a variety of tissue thromboplastin reagents were in common use, ranging from commercial extracts of animal origin to locally prepared reagents of animal and human origin. This was the worldwide position before a system of standardisation was initiated in Great Britain.

The British System

A scheme to introduce a standard system was begun in Manchester in 1962. Hospitals in the Manchester Region were supplied with a standardised phenolised human brain thromboplastin reagent for use in a Quick test technique and a method was recommended for the expression of results, as a ratio of prothrombin time of patients plasma to that of normal plasma (Poller 1964). Gradually, the scheme spread throughout the country and the thromboplastin became known as the Manchester Comparative Reagent (MCR) (Poller 1967). At this time the majority of hospitals in an area covering

approximately a quarter of the population of Great Britain were using the MCR in this way. Most of the remaining hospitals in the United Kingdom received the MCR against which they could compare their local reagent, whether home-made or obtained from a commercial manufacturer. The British Committee for Standardisation in Haematology adopted the MCR as the national reagent in 1969, and the term British Ratio was used for prothrombin time ratios obtained using the standard reagent.

In order to improve standardisation of reagent and reporting, batches of the MCR were subjected to independent monitoring. These were renamed British Comparative Thromboplastin (Poller 1970). The BCT reagent was supplied to those hospitals not using the routine MCR so that they could calibrate their locally prepared reagent or commercial reagent to a common standard. This calibration made use of an observation by Biggs and Denson (1967) and was based on the assumption that there was always a straight line relationship of prothrombin time ratios obtained for a set of patients plasmas with different thromboplastins. To perform the calibration, prothrombin ratios of at least twelve patients plasmas and six normal plasmas were obtained with the BCT and the local reagent. These ratios were plotted and a best fit line drawn by visual assessment of the data. The statistical validity of the procedure was discussed by Alderson et al (1970). The calibration could then be used to derive the British Corrected Ratio and meant that a result with a locally prepared reagent or a commercial reagent could be translated into the national scheme.

An important task was the monitoring of successive batches of the BCT in order to maintain continuity (Hill and Ingram 1973). These authors calculated that at least four normal and six patients plasmas would be required to detect the critical difference in sensitivity between two batches of BCT with an acceptable degree of certainty. This quantity of data was required from at least three centres. In practice four monitoring centres were asked to test five fresh normal and twelve patients plasmas each, as well as a lyophilised normal plasma. The maximum permissable difference in sensitivity between successive batches of BCT was a matter of clinical judgement. It was therefore the practice to accept a new batch provided that the 95% confidence limits for the ratio with the new batch lay within the range 1.9 - 2.1 for a ratio of 2.0 with the current batch. The use of the standardised reagent was supported by a series of quality control trials which allowed individual users of the reagent to compare their own performance with other participants (Leck et al 1976, Poller 1989). The preparation of the BCT and the monitoring exercise have been described by Stevenson (1978), together with the production of the first lyophilised form of the BCT. The first freeze dried BCT, batch 76/005, was prepared by the present author early in 1976 and was presented for the monitoring exercise in March of that year.

This was a significant development since it showed that the BCT could be produced in a stable form and still possess the character of the former wet reagent and its desirable properties, i.e., sensitivity to the coumarin-induced defect and ease of use in a well defined method, thereby satisfying the conditions proposed some ten

years earlier by Bangham (1966), Denson (1966) and Biggs and Denson (1966) when they laid down criteria for a standard thromboplastin preparation. Biggs and Denson (1967) proposed using, as a reference standard, a human brain extract with added calcium chloride, fibrinogen and factor V.

International developments

The International Committee on Thrombosis and Haemostasis (ICTH), in cooperation with the National Institute for Biological Standards and Control (NIBS & C), prepared a series of five lyophilised thromboplastins between 1967 and 1970. These were designed to represent the reagents and methods in popular use at this time. One of these was 67/40, a human brain extract as described by Biggs and Denson (1967) containing added fibrinogen and factor V. This type of reagent was referred to as a "combined" reagent to distinguish it from the "plain" reagents which contained only thromboplastin, e.g., 69/223, a human brain extract. The other three reagents were 70/115 and 70/178, rabbit brain extract, combined and plain respectively, and 68/434, bovine combined (Bangham et al 1973).

These materials were characterised in an international trial in 1974, carried out by an expert panel set up jointly by the ICTH and the International Committee for Standardisation in Haematology (ICSH). The results of this trial led to the establishment of 67/40 as the International Reference Preparation for thromboplastins, of the World Health Organisation, in 1976. This was the preparation

against which all thromboplastins had to be calibrated (WHO 1977). Each thromboplastin when calibrated against 67/40 was to be assigned a value known as the International Calibration Constant (ICC) which was the slope of the calibration line obtained with the IRP on the x-axis and the calibrated preparation on the y-axis. The ICC of 67/40 was defined as 1.0. Armed with an ICC value it was possible to convert the prothrombin time ratio obtained with a locally produced reagent into the value that would have been obtained using the IRP. This calculated value was termed the International Calibrated Ratio (ICR) (WHO 1981).

The WHO also established two secondary reference preparations, 70/178 (rabbit brain) and 68/434 (bovine brain) with ICC of 0.6 and 1.0 respectively. The reasons for setting up the secondary reference preparations were to prevent the primary thromboplastin being used up too quickly and to allow working thromboplastins to be calibrated against a reference preparation of a similar tissue extract. This scheme was not a success. Thromboplastin 67/40 was at the heart of the system and proof was needed of its stability. Ingram (1979) examined the evidence and suggested replacement of the IRP with a batch of the BCT. Also, 67/40 was a combined reagent. Lam-Po Tang and Poller (1975) showed, in an international survey, that plain reagents were in common use worldwide while the use of combined reagents was limited to Scandanavia and the Netherlands.

A further difficulty was that the WHO preparations were not freely available to commercial manufacturers of thromboplastins. Moreover, it gradually became apparent that the calibration model

was not adequate. In theory, the calibration line of prothrombin ratios should pass through the point 1,1 - this proved not to be the case especially when combined and plain thromboplastins were compared (Poller and Thomson 1969). A further problem was the noticeable curvature that showed when dissimilar thromboplastins were calibrated (Exner 1980, Loeliger and Lewis 1982). Kirkwood (1983) examined these shortcomings and showed that the existing statistical model fell short of the requirements for biological standardisation.

A further calibration study was then undertaken by ICSH and the Bureau Communautaire de Reference (BCR) of the EEC. Three new candidate reference preparations were developed, BCT 099 (human brain extract, plain), OBT/79 (bovine, combined) and RBT/79 (rabbit, plain) and a new calibration procedure was developed leading to revision of the WHO requirements (WHO 1983). This new statistical model used logarithms of prothrombin times instead of ratios and the linear relationship between thromboplastins was estimated by a procedure called orthogonal regression (Kendal and Stuart 1961). The preparation of BCT 099 is described in Chapter 7. The new technique was employed in 1983 to calibrate BCT 253, a further batch of the BCT. The object was to replace 67/40. This calibration was an extensive international exercise involving twelve European laboratories and five centres from the USA, Canada, South Africa and Australia (Thomson et al 1983). The calibration exercise was successful and BCT 253 was officially adopted by the WHO as the second International Reference Preparation. The two secondary IRP, 70/178 and 68/434 were also replaced at this time, by RBT/79 and

OBT/79 which had been calibrated in the earlier BCR exercise. The preparation of BCT 253 is described in Chapter 7.

Some new terms were introduced as a result of the revised procedure. The International Sensitivity Index (ISI) is the slope of the calibration line for any thromboplastin. It is obtained with logarithms of the prothrombin times of the primary IRP plotted on the vertical axis against the logarithms of the prothrombin times obtained with the calibrated thromboplastin on the horizontal axis. The International Normalised Ratio is the prothrombin ratio it is calculated would have been obtained if the primary IRP had been used in the test (INR = prothrombin time ratio^{ISI}).

The limited availability of the WHO preparations still raises difficulties and the BCR preparations, for which there is a charge, have not been available to Eastern European and developing countries. To remedy this situation, the ICSH set up a secondary IRP (BCT 441) prepared at the UK Reference Laboratory. This material was calibrated in 1985 (Thomson et al 1986) and is available, without charge, to designated national control laboratories in those countries where financial contraints apply. The preparation of BCT 441 is described in Chapter 7. The ISI values for the reference materials of human origin are shown in table 3.1

TABLE 3.1 ISI OF REFERENCE THROMBOPLASTINS

WHO 2nd primary IRP BCT 253	1.085
BCR secondary reference preparation BCT 099	1.048
ICSH reference preparation BCT 441	1.04

In practice, the current position is that the British System for anticoagulant control has become interlinked with the WHO system. The 2nd primary IRP (BCT 253) is one of three primary IRP; the others being RBT/79 (rabbit, plain) and OBT/79 (bovine, combined). The establishment of these three preparations means that any type of thromboplastin may be related to an IRP and the use of INR for reporting of results means that an internationally acceptable system is now in place.

CHAPTER 4 PARTIAL THROMBOPLASTIN

HISTORICAL REVIEW

In 1953, Langdell et al described a clotting time test which they called the "partial thromboplastin time" because the phospholipid thromboplastin clotted haemophilic plasma slowly, in comparison to the complete tissue thromboplastin of the prothrombin time test, which clotted haemophilic plasma in a normal time. The partial thromboplastin time test was shown to be sensitive to deficiencies of the known factors of the intrinsic and common pathways of blood coagulation (Brinkhous 1954).

The phospholipid requirement of the intrinsic activator is provided by the platelet membrane (see Chapter 2, above) and the elucidation of its nature has been the subject of considerable effort over many years. Some of the key events of this study are described in this chapter. The natural starting place for these studies was the use of lipids extracted from platelets.

Studies on lipids derived from platelets

In the late fifties and sixties a good deal of effort was put into a search for a single active fraction of platelet lipid which might substitute for the platelets in the clotting mechanism.

When platelet lipid extracts were fractionated on silicic acid columns (Rouser et al 1958, Marcus and Spaet 1958, Barkham et al

1961, Speer and Ridgway 1962, Marcus et al 1962) or by a combination of silicic acid column and paper chromatography (Troup et al 1960), or by column and counter current distribution techniques (Woodside et al 1964), it was found that clot-promoting properties were confined to the "cephalin" fractions (the ethanolamine [PE] and serine [PS] phospholipids). The choline-containing phospholipids and the neutral lipids were inert although combinations of lecithin (PC) with one or more of the cephalins did produce an active clot promoting preparation.

Apart from the common diacyl ester form, the glycerophosphatides occur in several other variations. One such, the monoacyl monoalk-1-enyl ether form, known as plasmalogen, was tested for activity by Rouser et al (1958), Zilversmit et al (1961) and Speer and Ridgway (1962). None of these groups were able to show any contribution to the coagulation sequence by plasmalogens.

Another variation in form occurs in platelet activating factor (PAF) which is 1-O-alkyl-2-acetyl-sn-glyceryl-3-phosphoryl choline. The alkyl chain is generally C_{16} or C_{18}. This compound unlike plasmalogen, is an extremely potent activator and can activate platelets independent of arachidonic acid metabolites or released ADP, and can cause shape change, aggregation and the release reaction at concentrations between 10^{-11}mol dm^{-3} and 10^{-10}mol dm^{-3}. Its structure was independently determined by Demopoulos et al (1979) and Benveniste et al (1979). PAF is produced by a variety of activated cells, for example, platelets (Chap et al 1981), monocytes (Benveniste et al 1972) and endothelial cells (Zimmerman

et al 1985). The bulk of the work on lipids in coagulation has, however, been restricted to the diacyl ester form of the glycerophosphatides.

Studies on nonplatelet lipids

Experiments were also performed using lipids obtained from sources other than platelets. Rouser et al (1958) and Rouser and Schloredt (1958) tried to relate structure to activity. Neutral lipids such as cholesterol and triglycerides were inactive, as were cerebrosides. Saturated fatty acids containing at least sixteen carbon atoms showed slight activity. A series of hydrolysis products of phospholipids showed no clot promoting properties. Individual phospholipids such as PC, SPH, PI, synthetic preparations and surfactants with structural similarities to phospholipids were also tested and shown to be inactive. Synthetic PE and PS were inactive as was liver monophosphoinositide. PE fractions from rabbit appendix, leucocytes, soya beans and human platelets all had activity (Platelet PE was most active). When the PE was hydroxylated with performic acid it was no longer active. Hydroxylated PE was soluble, so the implication was that there was a requirement for phospholipids to be in a dispersed colloidal particle in order to show activity.

Rouser and Schloredt (1958) studied the fatty acid composition of PE from various sources and showed that activity correlated with the degree of unsaturation of fatty acids. The suggestion was that more unsaturated fatty acids would give rise to a shorter molecule of greater diameter - a shape they believed to be more conducive to

forming a micelle. The active complex in the clotting sequence was visualised as a linkage, via divalent calcium ions, between an acidic phospholipid and a negatively-charged group on a clotting protein.

Wallach et al (1959) examined further the relationship between the colloidal state of purified phospholipids and their clot-promoting properties. This group used PE fractions from egg yolk, obtained by silicic acid chromatography. Column eluates with differing degrees of saturation were studied in a clotting system comprising purified factor VIII, factor V, calcium, magnesium and the test lipid. Etheral solutions of phospholipids were injected into the system and the solvent removed by bubbling nitrogen through the suspension.

The more unsaturated preparations formed stable suspensions at neutral pH. A pH of 8 was necessary to prepare a stable emulsion of the more saturated material, and synthetic dimyristol-PE could be suspended only at a pH of 10. With a rise in pH of the suspending medium there was a gradual increase in clotting activity to pH 8, then potent activity to pH 10 after which the activity was lost. The clot promoting activity of these PE fractions therefore appeared to depend mainly on physical characteristics of the emulsion. This in turn was shown to be governed by the degree of unsaturation of the constituent fatty acids, temperature, pH and ionic composition of the aqueous medium in which it was suspended. It was concluded that clot promoting PE preparations were micelles in the form of

bimolecular leaflets of a definite size, thickness and a unique surface configuration.

Meanwhile, Rouser et al (1961) devised a technique which separated PE from PS more efficiently. Using this procedure, Marcus et al (1962) found the separated PS to be the most active group of phospholipids. Later, Marcus (1965) and Marcus et al (1966) found that isolated platelet membranes were more efficient than platelet granules in certain clotting systems. This reinforced the orginal proposal of Surgenor and Wallach (1961) that the reactive site of the platelet in coagulation is orientated towards the plasma, and is perhaps in the platelet membrane.

Early observations on the charge of phospholipids and its relation to clot promoting activity

In the earlier studies of Rouser and Schloredt (1958) and Wallach et al (1959), attention was directed primarily toward the hydrophobic region of the phospholipid. Bangham (1961b) examined the polar region of the phospholipids. Egg PC was mixed in varying amounts with dicetyl phosphate in choloform, taken to dryness and emulsified in tris buffer. The zeta potential of these mixtures correlated with their activity in the Russells Viper venom test (RVV is able to directly activate factor X, bypassing both the extrinsic and the intrinsic pathways). When the electrophoretic mobility of various emulsions used in the test system was measured, it was found that the more negatively charged suspensions produced shorter clotting times. There was excellent correlation between the

activity of PC: dicetyl phosphate mixtures and other known clot-promoting materials. For example the electrophoretic mobility of a brain lipid extract (Bell and Alton 1954) resembled that of the active PC: dicetyl phosphate and both gave a clotting time of 8 seconds. Platelets and a PC:PE mixture behaved similarly.

These observations were confirmed and extended by Papahadjopoulos et al (1962), working with phospholipids prepared from bovine brain and egg yolk. Four combinations of phospholipids were used in a wide range of concentrations - PS in PE, PS in PC, PA in PC and PE in PC. Maximal clot-producing properties in a standard clotting assay were found with mixtures which showed an electrophoretic mobility of $-4.5 \times 10^{-4} cm^2 sec^{-1} volt^{-1}$. Thus there was an optimal negative surface charge at which phospholipids exhibited maximal clot-promoting properties and the clot-promoting properties of various phospholipid mixtures were clearly correlated with the surface charge of the micelles they formed in aqueous media.

Recent Studies on Membranes in Blood Clotting

The work of Zwaal et al (1973), Zwaal et al (1975), Otnaess and Holm (1976), Chap et al (1977), Zwaal et al (1977), Schick (1978, 1979) has shown that the plasma membrane of red blood cells and platelets is based on an asymmetric phospholipid bilayer whose leaflets have a distinctly different composition. The choline containing phospholipids, particularly SPH, and also substantial amounts of PC are located in the outer layer whereas a large

fraction of PE and practically all the negatively charged phospholipids - PS and PI - are confined to the inner (cytoplasmic) surface.

A number of methods have been employed to investigate plasma membrane phospholipid asymmetry. One approach has been to use external labelling probes. E.g., in the work of Schick when trinitrobenzenesulphonate (TNBS) was employed. This compound reacts with primary amine groups and is thus able to form stable covalent derivatives with PE and PS. Because of its highly polar nature TNBS does not penetrate the surface of intact cells. In his studies Schick incubated washed cells with TNBS then extracted labelled phospholipids and separated them by thin-layer chromatography. Quantitation was by assaying phosphorus and the percentage of PE or PS that had formed a derivative with TNBS was calculated. Schick (1976), using this method, showed that only small amounts of PE and no PS were exposed on the surface of platelets. A second label, 1-fluro-2,4-dinitrobenzene (FDNB) (Schick 1978) was employed to confirm that aminophospholipids are located primarily in the inner platelet membrane bilayer. FDNB, like TNBS, forms stable derivatives with PE and PS but is able to penetrate the surface of intact cells.

An alternative approach, employed by Zwaal's group, was to use purified phospholipases to degrade the surface of cell membranes, thereby building up a picture of the distribution of phospholipids. These enzyme molecules are too large to penetrate the cell membrane. For example phospholipase A_2, from Naja naja venom, and

sphingomyelinase, from Staphylococcus aureus, were used to degrade the surface of pig platelets. A purified preparation of phospholipase C from Bacillus cereus was also employed (Chap et al 1977). The results of this study differed from the earlier results of Otnaess and Holm (1976). The differences may be due to the fact that Otnaess and Holm did not inactivate phospholipase C before lipid extraction so that the enzyme continued to hydrolyse the extracted lipids, and that there may have been a difference in specific activity in the sphingomyelinase preparation, used in the two studies.

The important point, however, is that the results of the enzyme study are in good agreement with those from the studies employing labelling methods, and both indicate that there is an asymmetrical arrangement of phospholipids in the platelet membrane with PE and PS located primarily in the inner bilayer.

Zwaal et al (1977) prepared liposomes having the same composition as the outer membrane layer of either platelets or erythrocytes. These did not shorten the clotting time in a one-stage prothrombinase assay employing Russell's viper venom. In contrast, liposomes with the composition of the inner surface considerably reduced the clotting time. Also, intact erythrocytes and resealed ghosts had no procoagulant activity, whereas preparations with the cytoplasmic surface exposed, e.g., non-sealed ghosts and inside-out vesicles, markedly reduced the clotting time.

It has been pointed out (Schick 1979) that the characteristics of the phospholipase C (Clostridium perfringens) - induced platelet release reaction are identical to those induced by thrombin. It is possible that PC may mask a membrane protein that can serve as an active site for platelet activation, or even be a receptor itself. Fiedel et al (1976) showed that anti-PC antibodies isolated from myeloma paraproteins can inhibit platelet aggregation. Also, Cooper et al (1974) demonstrated this inhibition by immuno-globulin from patients with myeloma. Another interesting observation (Lubin et al 1981, Chin et al 1981) concerns alterations in phospholipid orientation which occur in irreversibly sickled cells or in reversibly sickled cells that only sickle in anoxic conditions. The process of sickling is accompanied by an increased exposure of PE and PS at the cell exterior, at the expense of some PC, presumably by the process known as flip-flop. During sickling, the cells cause a reduction in clotting time when used as a source of lipid in a one-stage prothrombinase assay. When reversibly sickled cells resume their normal biconcave shape following re-oxygenation, they have no effect on clotting time. The flip-flop mechanism is not well understood, but may well require some sort of control by trans-membrane proteins (see Chapter 2).

In 1964, Papahadjopoulos and Hanahan showed that a complex of phospholipids, factor Va and factor Xa was responsible for the conversion of prothrombin to thrombin. None of the components of this complex, alone, could perform the function of "prothrombinase".

In 1967, Hemker and Kahn showed that the activation of factor X is achieved by a complex of phospholipids, factor VIIIa and factor IXa. Again the "tenase" function can not be performed by any of the components of the complex alone.

These two complexes have much in common. They each consist of three components - phospholipids, enzyme (Xa or IXa) and an "auxiliary" protein (Va or VIIIa). Rosing et al (1980) and Van Diejen et al (1981) have shown that the enzyme alone is able to convert its product at a very slow rate. Addition of the auxiliary protein increases the rate of conversion by one thousand times, presumably by directing the vulnerable site of the substrate towards the active site of the enzyme. The phospholipids decrease the apparent K_m of the substrate by a thousand times, causing enzyme saturation at much lower substrate concentration at the lipid surface. These workers believe that the non-enzyme components in the complexes can produce an increase in the actual reaction velocity of up to 10^6 times.

Only certain mixtures of phospholipids show an optimal procoagulant activity. Apart from a net negative charge it is clear that fluidity is important. Tans et al (1979) have demonstrated that phospholipids in the gel state show very little activity.

As discussed above, in both platelet and red blood cell membranes, PS is preferentially located on the cytoplasmic side of the bilayer. This is the reason why lysed platelets and red cells which expose cytoplasmically orientated lipids to the clotting

factors, are much more active than intact cells when used as a lipid source in the prothrombinase complex (Zwaal et al 1977). A similar phenomenon was observed when other cells, e.g., leucocytes and endothelial cells were used as a source of lipids (Zwaal and Bevers 1983). This suggests that in these cells also, negatively charged phospholipids are absent from the outer cell surface.

Zwaal and Bevers (1983) and Zwaal et al (1980) have shown that after stimulation by a mixture of collagen and thrombin, phospholipid orientation in the platelet plasma membrane is radically altered. Before activation, very small amounts of negatively charged PS may be found at the exterior, but, following activation PS and PE are greatly increased. This leads to increased binding of the components of the prothrombinase complex, resulting in a higher prothrombin-converting activity. The appearance of PS at the outer surface cannot be explained by just the release reaction, which involves fusion of intracellular membranes with the plasma membrane, since no increase in exterior PS is observed when platelets are activated by either collagen or thrombin alone. Zwaal and Hemker (1982) suggest that the combined action of collagen and thrombin produces sufficient perturbation of the membrane to allow flip-flop of phospholipids. PE and PS may flip-flop to the outside while PC may flip-flop to the inside layer, in a way similar to that observed for sickle cells under anoxic conditions.

Platelets also function as a lipid source for "tenase". Here too, the combination of collagen and thrombin is much more effective than thrombin alone. The rise in "tenase" activity seems to precede

the rise in prothrombinase activity. Walsh (1978) observed that collagen-activated platelets enhance factor X activation prior to stimulating prothrombin activation.

Zwaal and Hemker (1982) suggest that the translocation of PS occurs at distinct loci in the membrane. This will lead to local high concentrations of PS which facilitate the action of "tenase". Due to lateral diffusion of the phospholipid in the plane of the membrane the PS concentration in these patches will be diluted to become more favourable for prothrombinase.

CEPHALIN AS A LABORATORY REAGENT

In the coagulation laboratory the APTT has a number of functions. It is used to screen intrinsic coagulation defects, i.e., to identify specific clotting factor deficiencies, to detect the presence of inhibitors and also for monitoring heparin therapy. A great variety of methods and reagents is available with varying sensitivities to the different defects and inhibitors. The position is not improved by the fact that no international reference preparation exists, nor any system of calibration of APTT reagents or reporting of results.

Poller and Thomson (1972) reported on the need to standardise both reagents and technique. Subsequently, reports from a large number of workers have confirmed the variability in performance of commercial and locally produced APTT reagents in the detection of intrinsic clotting defects (Sibley et al 1973, Poller et al 1976,

Shapiro et al 1977, O'Brien et al 1981). Hoffman and Meulendijk (1978) went so far as to say that none of the available commercial partial thromboplastins was suitable for use as a standard.

Consideration of the APTT method recommended by the UK Reference Laboratory for Anticoagulant Reagents and Control (Thomson 1980), which is designated the Manchester APTT method, will demonstrate some of the areas where attention to standardisation of reagents and technique is vital. The test system is buffered to a physiological pH of 7.35 and activation of the contact factors is achieved by incubation for ten minutes in the presence of kaolin. Originally, the lipid employed was of human brain origin, extracted by ether from an acetone-dried preparation of brain powder. The etheral extract was dried on to the walls of a round-bottomed flask, then shaken in buffer to suspend the lipids. Fig 8.5 shows an electron micrograph of the resultant liposome suspension and details of the lipid composition are given in Chapter 8.4.

The areas of contention in the APTT test concern buffering, choice of activator and time of activation, and the composition of the lipid reagent. Other issues lacking agreement concern collection of plasma, the question of plasma dilution and calcium concentration. It appears that buffering is favoured by the majority of manufacturers although the choice of buffer is not revealed.

Activators, on the other hand, are generally disclosed. Kaolin has proved to be one of the most effective and is the one which has

been incorporated in the Manchester APTT method. Both silica and ellagic acid are to be found in a number of commercial reagents and are favoured for use in automated techniques on account of their lower opacity. Margolis (1958 and 1961) first described the use of kaolin and of silica in a partial thromboplastin time test, while Ratnoff and Crum (1964) first drew attention to the use of ellagic acid. A possible physiological activator, suphatides, was more recently described by Fujikawa et al (1980) and further characterised by Tans and Griffin (1982). This activator has already been incorporated in a commercially available APTT test system (Behringwerke 1984).

On the question of the lipid reagent, a variety of sources of lipids have been extracted, e.g., animal brains, platelets, and soya beans. As a result there are differences in the actual individual lipid classes represented in the reagents, as well as in total lipid present. This topic is dealt with at length below (see Chapter 8).

Use of the APTT in the diagnosis of intrinsic clotting defects

Reports describing the use of the APTT may be divided into those dealing with single laboratory data and those concerned with multi-centre surveys. Poller and Thomson (1972), Sibley et al (1973), Babson and Babson (1974), Hoffman and Meulendijk (1978) are examples of the first group which showed the variability in performance of commercially available partial thromboplastins. Some multicentre reports such as Poller et al (1976) and Koepke (1975, 1977) give a wider view and do not suffer from any local bias or

varying expertise that might colour single centre studies. The study of O'Brien et al (1981) is an example of a highly specialised study which dealt only with the diagnosis of mild haemophilia and was performed in expert centres.

It is common to all studies mentioned, whether concerned with sensitivity of APTT methods to congenital or acquired coagulation defects, factor VIII determination or any other intrinsic factor measurement, that they demonstrate the varying sensitivities of all the APTT reagents studied. Manucci et al (1979) reported the variation in response to the circulating anticoagulant of lupus erythematosus shown by a number of commercially available reagents. This is dealt with in greater detail below (Chapter 8.5 and 8.13).

Use of the APTT in the Control of Heparin Therapy

Heparin is administered in conventional (or high-dosage) levels, resulting in blood levels greater than 0.2 units cm^{-3}, or in low-dosage levels, resulting in blood levels of 0.01 to 0.2 units cm^{-3}. The APTT is the most commonly used method, worldwide, for monitoring heparin. Even so, the sensitivity to heparin varies considerably in the different reagents. The method should be adequately sensitive to heparin over a wide range of concentrations and should show a linear relationship with concentration. Triplett et al (1978) reported that in a proficiency survey involving over 2,000 hospitals in the United States, there was considerable variation of sensitivity in measurement of conventional dose heparin and the non-linearity of a number of commercial reagents to

increasing concentrations of heparin up to 0.8 units cm^{-3} was shown by Shapiro et al (1977).

Poller et al (1980) organised a study, involving over 300 hospitals in the UK and overseas, to assess the sensitivity of APTT tests used to measure the anticoagulant effect of in vitro additions of differing amounts of heparin to a standard plasma. Participants were asked to reconstitute four coded vials of plasma with four different reconstitution fluids. In fact, the plasma was all from the same donor and the reconstitution fluids were, respectively, water and heparin solutions (0.03 u cm^{-3}, 0.06 u cm^{-3} and 0.12 u cm^{-3}). In this way it was possible to test the same plasma with a wide range of heparin concentrations.

Tests were carried out with local routine APTT reagent and technique, and also with the Manchester APTT reagent and technique. A sensitivity ratio was calculated, i.e., APTT of heparin containing plasma divided by APTT of normal plasma. The results emphasized the wide discrepancies which exist between commercial APTT reagents in their sensitivity to the anticoagulant effect of in vitro heparin. Some thirteen reagents from commercial sources were used by participants in this study. None of these showed as great a sensitivity as the Manchester Reagent, even though the latter was not familiar to over half the participants. Eight of the commercial reagents failed to detect the lowest heparin concentration.

Taberner et al (1979) evaluated the sensitivity of the Manchester reagent in the detection of circulating heparin,

administered in the low dose regime to patients undergoing gynaecological surgery. The Manchester APTT method appeared to offer a much simpler and more effective method of control than methods designed to assay the concentration of free heparin, i.e., anti-Xa test (Denson and Bonnar 1973) and a chromogenic substrate assay (Teien et al 1976).

Poller et al (1982) confirmed the usefulness of the APTT over the anti-Xa assay in a study which examined the effects of low dose heparin in patients undergoing hip surgery. The study was performed to determine the degree of anticoagulation required to protect these high risk patients from post-operative deep vein thrombosis. As discussed in Chapter 2, Hemker (1987) also prefers the use of the APTT for detection of circulating heparin.

Barrowcliffe and Gray (1981) demonstrated many of the technical variables that exist in the APTT in the detection and measurement of heparin. They showed that the contact activator was important. Reagents that employed ellagic acid were noticeably less sensitive to heparin than kaolin-activated reagents. This was tested by using the two activators with the same phospholipid reagent. Time of activation was also significant. It appeared that ten minutes was optimal giving full activation of both heparinised and non-heparinised plasma, which is in agreement with the ten minute kaolin activation time recommended with the Manchester APTT reagent. Buffering increased the sensitivity of the heparin measuring system. These authors also found an increase in sensitivity with dilution of the lipid reagent.

THE NEED FOR STANDARDS

From the above, it is clear that standardisation of the APTT is urgently required. Standardisation must be directed at the phospholipid reagent, since this is central to the performance of the test system.

In Chapter 8, a series of experiments is described which show the differences between some commonly used APTT reagents in testing a variety of normal and abnormal plasmas. The ultrastructure and the lipid compostion are examined with a view to correlating clotting performance with some physical or biochemical feature of the reagents. A range of liposomes of varying composition is then examined to determine whether it is possible to manufacture a liposome that might provide an optimal surface for clotting and that might maximise the sensitivity of the reagent for all its laboratory uses. If this proves possible it may be of use in eventually formulating a lipid reagent acceptable as an International Reference Preparation (IRP).

CHAPTER 5 FREEZE-DRYING

Freeze-drying, or lyophilisation, may be described as the removal of water from a frozen material by the sublimation of ice. The process may be divided into several stages.

Stage 1 - Pre-freezing. The material to be freeze-dried is frozen, generally at atmospheric pressure. During this procedure, water separates from solution to form pure ice crystals. The frozen material is then taken to stage 2.

Stage 2 - Sublimation. The frozen material is placed in a chamber maintained at low water vapour pressure, under which conditions ice will sublime directly from solid to vapour phase. A correct balance of heat input and vapour removal is important at this stage to support sublimation. Excessive heating causes melting of the material with disastrous results, while insufficient heating will permit the system to equilibrate, with the result that the frozen material will not sublime and the process will come to a stop. The water vapour evolved from the drying material is removed by condensation on a cold trap, by absorption by a desiccant or by pumping. When sublimation, or primary drying, is complete, a quantity of moisture is still retained (this may be as much as 6 or 7%), which will not be removed by prolongation of this stage.

Stage 3 - Desorption, or secondary drying. The remaining water from stage 2 is removed in the desorption stage. Heat is still applied to the material which is at very low vapour pressure and

exposed to a desiccant, such as phosphorus pentoxide. Alternatively, lowering the temperature of the cold trap may improve the efficiency of this stage in the process. Several hours or days may be necessary to bring the residual moisture to the optimal level required for storage.

HISTORICAL REVIEW

Rey (1978) discussed the method used by the Incas, in pre-Columbian times, to prepare food for storage. The method apparently involved storing food at high altitude so that it froze and was then progressively dehydrated by sublimation of ice, by radiant heat at the reduced water vapour pressure of the rarefied atmosphere. These are the same conditions that obtain in a present-day freeze-drying plant.

The first modern reported use of freeze-drying was by Altmann (1890) who dried frozen tissues in a vacuum desiccator at -20°C for preparation of histological specimens. The first patent with thorough descriptions of the subject was that of Elser (1934) which described a method for drying under a vacuum of a few mb and a cold trap of solid CO_2. In the following year Elser et al (1935) described the introduction of heat into the system to supply heat of sublimation.

The first commercially practical design, using a cold trap of methyl cellosolve, containing dry ice, was described by Flosdorf and

Mudd (1935). The same low temperature bath was used to freeze the specimens.

Greaves and Adair (1936) described the "frothing" phenomenon which occurs on evaporative freezing of materials for freeze-drying. Rapid evaporation under vacuum causes loss of 12.5 to 20% of water, causing the remaining solution to freeze. Frothing occurs as dissolved air is released from solution. Greaves and Adair (1939) described their mechanically refrigerated freeze-drying plant and made a full analysis of the physical principles involved, on a sound scientific and engineering basis.

The second World War brought a demand for large scale production of dried plasma. Flosdorf et al (1940) designed a production plant for this purpose, using a large mechanical pump to substitute for a refrigerated trap or desiccant. The condensed water vapour had to be removed from the pump oil by high speed centrifugation. Eventually, large mechanically refrigerated condensers were preferred for removal of water vapour. Strumia and McGraw (1943) produced a small size drying unit, with facilities for shell freezing twenty-four bottles of plasma, by rolling them in a refrigerated bath. The plasma bottle was supported on rollers and rotated at about 30 revolutions per minute, with its axis horizontal and partially immersed in a cold fluid. Their plant used a mechanically refrigerated cold trap.

Greaves, meantime, in Great Britain, was examining the same problems. He introduced the vertical spin freeze, in which plasma

bottles were spun at high speed, in a vertical position, in air, at -18°C, to obtain shell freezing with very small crystal size. Greaves also made the discovery that a superior rapid freeze, without frothing, could be obtained by evaporative cooling under vacuum, simultaneous with the high speed vertical spin. The original plant was in operation in 1940 and Greaves (1946) reported the theory and practice of the work.

THE SUBLIMATION STAGE OF FREEZE-DRYING (PRIMARY DRYING)

The aim of this stage is to remove ice with the minimum damage to the product. It may be further divided into a number of phases.

(i) the initial phase corresponding to the start of heating. Within a relatively short time the evaporation rate rises rapidly to a maximum value.

(ii) the sublimation phase. The interface between dry and frozen substances recedes from the surface to the interior of the product. The evaporation rate decreases rapidly because the resistance of the pores to heat flow and to vapour flow increases rapidly with the distance separating the interface of sublimation from the free surface of the product.

(iii) the desorption or secondary drying phase. The evaporation rate decreases rapidly and approaches zero.

A plot of the evaporation rate against time would also serve as a presentation of mass transfer expressed as the weight of the sublimed or desorbed water per unit of time during the various phases of treatment. Mass transfer is at all times in equilibrium with heat transfer and both result from the action of temperature and pressure gradients, and depend on the thickness of product and its physical characteristics.

The parameters which enable us to influence the rate of heat and mass transfer can be divided into two groups:-

(i) those integral to the substance which define its structure, i.e., granulation of the product, number, dimensions and orientation of the pores. These depend on the method of preparation and freezing of the material.

(ii) parameters external to the product, i.e., temperatures, partial pressure of vapour and total pressure during freeze-drying. These depend on the freeze-drying plant.

Whatever the state of the substance being dried there exists an 'interface' or 'front of sublimation' between dry and frozen substance, which is shifting progressively and which, at a given time, has a temperature T_1 with a corresponding saturation pressure of the vapour P_1. The mass transfer, MT, results from the migration of the vapours through the pores of the dry substance under the action of the difference in vapour pressure. P_1 minus

P_2 is at most equal to the total pressure, i.e., the value of the vacuum over the dry surface of the product. The mass transfer, MT, will be the more intense when $P_1 - P_2$ is greater provided it is balanced, at every instant, by an identical heat transfer HT. Consider the transfer of heat by conduction across the substance being dried, under the action of the temperature difference $T_2 - T_1$. (T_2 being the temperature at the surface of the substance in contact with the heat source). Since in the freeze-drying plant in use at the UK Reference Laboratory, this source is the shelf, heat transfer takes place through the frozen substance. In this case the temperature, T_2, the temperature at the shelf/product interface must be less than T_f, the limiting temperature, the temperature of incipient melting. The only parameter external to the substance which can be manipulated is P_2, the maximum vacuum, and this does not influence the heat transfer, HT, through the frozen layer of the substance. As HT decreases in proportion to the increase in T_1, it is therefore advantageous to fix P_2 as low as possible. The mass transfer is reduced with increasing depth of the interface and in compensation, the value of the interface temperature T_1 increases. The heat transfer increases with growing depth of the interface, X, but decreases with increasing T_1. As a result, the rate of drying decreases rapidly towards the end of the sublimation period when T_1 approaches T_2.

With regard to structural parameters - these only exert influence on the mass transfer, but since X is of high value, this transfer becomes the limiting factor, especially toward the end of

sublimation and the structural parameters eventually exert influence on both the rate and the duration of drying.

Some considerations in equipment design

The condenser is one of the most important components in a freeze-dryer. Despite the significance of the subject, surprisingly little has been published on condenser design. Triggs (1944) designed a hollow refrigerated condenser in which the ice, as it formed, was continually scraped off and removed through a vacuum lock. This design was an attempt to overcome the problem of the insulating effect of the growth of the condensing ice layer. The heat-flow resistance due to the ice layer on the condenser is proportional to its thickness and inversely proportional to its thermal conductivity. Condensers are designed from the point of view of providing the maximum area for the depositon of ice, so little attention is given to the question of heat conduction through the ice layer.

Rowe (1976) discussed the efficiency of mechanically cooled condensers and believed that the temperature of the condensing surface must be maintained lower than that of the product and low enough to achieve an accommodation coefficient near to unity, i.e., molecules striking the condenser must be trapped and rapidly brought to its temperature so that further molecules may then be accommodated and so on. He believed that for the majority of purposes, a condenser temperature of $-45^{o}C$ to $-50^{o}C$ should be adequate. Shumsky (1958) devised a theory for sublimation

condensers and presented formulae for calculating the required surface area, ice distribution and heat transfer. Ciborowski and Surgiewiez (1961) also examined the problem paying particular attention to freeze-drying in fluidised beds.

In 1966, deRoissart and Laederich devised a formula for determining the ice capacity and layer thickness on condensers cooled by mechanical refrigeration, solid CO_2 or liquid nitrogen. They introduced the term "temperature deficit", i.e., the difference in temperature between the ice surface and the condenser surface. Using their equations, it is possible to calculate that at the end of sublimation and allowing a temperature deficit of 170°C in the case of liquid nitrogen cooling, the estimated ice layer thickness should be 130 mm. For a mechanically cooled arrangement the temperature deficit might be 20°C resulting in an ice layer thickness of 20 mm.

Rowe (1964b) suggested guidelines to the design and operation of mechanically refrigerated condensers. He suggested that the condensing system should comprise an array of coils or plates, that the temperature at the ice layer surface should be -40°C, at which point the saturated vapour pressure of ice would be some 0.13 mbar. The vacuum pump should maintain a partial pressure of about 0.013 mbar. Rowe concluded that the surface area of the condenser should equal that of the shelves in the drying system, although as little as half of this surface may be adequate (see below, chapter 6). A further problem discussed by Rowe is that of uneven frosting in condensers. No overall solution has been found to this problem.

Mellor and Munns (1968) described a system of baffles designed to produce uniform build-up of ice in one of their machines, but made no suggestion about condenser surface area.

These findings and observations were a major influence in the design of condensers for the freeze-drying machine, which was custom built for the UK Reference Laboratory for Anticoagulant Reagents and Control, and is described in Chapter 6.

The mechanically cooled condenser was of copper tube, coiled to allow the condensation of some 18 litres of ice. The liquid nitrogen cooled trap was of a different design, with similar capacity but smaller surface area. A primary objective in the design of both devices was that there should be minimum impedance to vapour flow into the trap, while at the same time there should be maximum capacity for condensate.

Resistivity control of heating in sublimation

Typical values of resistance for aqueous solutions are about 10 - $10^2\Omega$ but on freezing the resistance may change to $10^6\Omega$ or more. It is possible to determine the resistance of the material when it is just frozen. The resistance of the material can then be monitored throughout the sublimation phase and heaters can be switched off if the resistance falls below the preset value. The switching of the heating can be made automatic, by the use of a suitable relay in the heating circuit. The normal manual control of heating is, of course, still necessary after sublimation, when the

resistance will be permanently high. By use of the resistivity control, the correct quantity of heat may be applied, always maintaining T_2 just below T_f for the substance being dried.

THE DESORPTION STAGE OF FREEZE-DRYING (SECONDARY DRYING)

Freeze-drying does not stop after the sublimation of all the frozen water. All products retain, by adsorption, a quantity of water which endangers the good conservation of the material - there may be up to 7% of moisture remaining after sublimation. Secondary drying is intended to reduce this to an optimum value for stability - between 0.5% and 2.5%.

Proper desorption is very difficult owing to the very low vapour pressure of adsorbed water - generally what is required is an increase of temperature to as as high a level as will be tolerated by the material, and a high vacuum. The moisture trap in secondary drying is usually a trap containing a desiccant rather than the cold trap of primary drying. Phosphorus pentoxide is the most commonly used, and the most effective desiccant - it reacts with water irreversibly, to form a series of phosphoric acids. It is an unpleasant material to handle, both in its dry and quenched states.

Damage from freeze-drying

There are four stages in the freeze-drying process where injury can occur - during the initial freezing, during drying, during the

subsequent storage or on reconstitution. This area was discussed in detail by Stevenson (1978).

Damage during freezing

Damage may be prevented or alleviated by the addition of cryoprotectives. Unfortunately, the most effective additives such as dimethyl sulphoxide and the glycols are not of much use in preparations for freeze-drying since they are not easily sublimed.

Damage from drying

Dehydration takes place during freezing. This dehydration is said to be less damaging to microorganisms, for example, than the dehydration that occurs during freeze-drying. This observation has been taken as support for the idea that freeze-drying removed a portion of water that was "unfreezable" or "bound".

Bound water has been "measured" in terms of so-called unfreezable water. While it is true that in any cooling experiment, especiallly one in which some or all of the solutes do not crystallize, a certain proportion of water does not appear to freeze, the reason is not that this water is prevented from freezing, because it is bound to some non-aqueous species. Franks (1986) discussed the cooling of a dilute sucrose solution. Ice begins to form at the equilibrium freezing point (which depends on the initial concentration). Further cooling leads to an increase in the concentration and the viscosity of the solution. At -32^{o}C the

concentration is equivalent to 0.5 g water (unfrozen) per gram of sucrose. The time required by a water molecule to diffuse through a given distance depends upon the viscosity. At -32°C this is more than 3 years/μm. Water is therefore not unfreezable, but unfrozen on the time scale of the observation. It is not bound. Even pure water would behave similarly, that is, at -150°C ice crystals would grow at 0.3 μm per year.

During drying, denaturation can take place in the still frozen, though undried, portions of the specimens, due to the concentration of solutes during freezing. The rate at which adverse reactions proceed will be increased by exposure to higher drying temperatures. This suggests that susceptible materials should be dried at temperatures low enough to reduce rates of reaction, and low enough to eliminate all liquid phase, since any liquid phase present, from eutectic solutions will enable reactions to continue.

A further mechanism of drying damage may be from the recrystallization of salts or hydrates formed from eutectic solutions. Rey (1964) and MacKenzie and Luyet (1964) studied the freeze-drying process through time-lapse photomicrographs of the progression of drying in a thin film of solution. There are a number of interesting observations on their work. 1) as the drying boundary passes through a large crystal of ice, the sublimation surface appears to be parallel to the crystal planes. 2) individual ice crystals dry independently of each other. Isolated crystals may remain undried after surrounding crystals have sublimed. 3) when solutions are drying, the drying boundary is not a sharp interface,

but a zone of uniform width, well demarcated on either side from the frozen and the dried portions of the specimen. There is great activity within the drying zone, suggesting recondensation and recrystallization. The salt or hydrate crystals left behind are related in size to the drying temperature at the moment when that particular area was drying. Raised temperature produces larger crystals. Considerable structural damage may result from the crystallization of these salts and hydrates - once again the suggestion is that low drying temperatures should be employed when drying injury is a problem.

Damage during storage following drying

There are a number of deleterious reactions that can take place during the storage of dried materials at room temperature. Hannan and Lea (1952) emphasize the reactions between proteins and reducing sugars as being very important. This reaction may be minimized by reducing the amount of carbonyl-containing compounds, such as glucose, in the medium and by adding carbonyl acceptors such as sodium glutamate. This 'browning reaction' can also occur between organic acids and ascorbic acid, proteins and other amine groups, in fact, almost all the chemically reactive constituents present in the specimen. These reactions will be inhibited at low moisture levels.

The other main class of adverse reactions involves oxidation, which is of the greatest significance in dealing with thromboplastins. The exclusion of oxygen from containers of dried material is therefore of considerable importance - in freeze-drying

of biological materials, vials may be sealed after introduction of a partial pressure of nitrogen or argon. The procedure for this step is described in some detail below (Chapter 6).

Damage from reconstitution

Even though the exact amount of water removed by drying is replaced, it cannot be replaced uniformly. This means that as reconstitution is going on there will be substantial concentration gradients, transport of solutes throughout the specimen and osmotic imbalances which may lead to a specimen that differs quite radically from the fresh material. This aspect of the work is not discussed in any detail in the literature.

CHAPTER 6 METHODS AND MATERIALS

6.1 SEPARATION OF LIPID CLASSES BY THIN LAYER CHROMATOGRAPHY

No single one-dimensional method was able to resolve the lipids of interest. Accordingly, a number of thin-layer chromatography (TLC) methods were employed, which are described below.

For calibration purposes a standard phospholipid mixture was prepared which comprised di-PG, PA, PG, PS, PE, PI, PC, SPH and lyso-PC in the proportions shown in table 6.1.

TLC plates were activated at 120^{o}C for 15 minutes before use. To maintain saturated conditions in the chromatography tank, a piece of filter paper (Whatmans no 3) was cut to 22 x 22 cm and placed against one wall. Fresh solvent mixture was added to the tank by pouring down the paper, the lid replaced and the tank allowed to equilibrate for at least 30 minutes before use.

Lipids were extracted from test samples of tissue thromboplastin, partial thromboplastin or liposomes by the method of Bligh and Dyer (1959). Aliquots of lipids, dissolved in chloroform or chloroform:methanol (95:5 v/v), were stored in glass vials at -40^{o}C until required for TLC.

TABLE 6.1 COMPOSITION OF STANDARD LIPID MIXTURE

Lipid Class	% by weight
diPG	1
PA	1
PG	2
PS	1
PE	6
PI	4
PC	53
SPH	24
lyso-PC	8

Visualisation of separated spots - Four methods were employed:

1 Periodic acid - Schiff stain (PAS)

The dry chromatogram was sprayed with sodium periodate (1% aq) and allowed to stand for 5 minutes. It was then dried under a stream of warm air, and placed in a perspex box (235 x 235 x 190 mm) flushed with sulphur dioxide. After the plate was cleared of excess iodine, it was removed from the box, lightly sprayed with Schiffs reagent and returned to the SO_2. PG stained immediately. Several hours were required before all the phospholipids stain to sufficient intensity for quantitation by densitometry. PA does not stain ty this method.

2 Charring at 130°C for 30 minutes on a Desaga HP Thermoplate (Uniscience Ltd, Cambridge), following spraying with 50% methanolic sulphuric acid. This method reveals all the organic material on the chromatogram.

3 Charring at 130°C for 10 minutes following a dip in 0.82 mol dm^{-3} phosphoric acid containing 0.17 mol dm^{-3} of cupric

acetate. This method produced more dense staining of the neutral lipids than did method 2.

4 Ninhydrin staining of aminophospholipids. A fresh solution of 14 mmol dm^{-3} ninhydrin in 9:1 (v/v) acetone:lutidine was sprayed on to the dried TLC plate and the layer allowed to dry before respraying. The plate was then heated at 100°C for 5 minutes. Amino-containing phospholipids appeared as purple spots (Marinetti 1964).

TLC system 1

A silica gel plate (250μ layer) incorporating ammonium sulphate (Camlab Ltd, Cambridge) was run in the solvent system chloroform:methanol:water (65:25:4) by volume. Lipid standards, individually, or in the mixture described, were applied 15 mm from the bottom of the plate. Development under saturated conditions was stopped when the solvent front reached 140 mm. Visualisation was by method 1.

This system was developed for routine use in the UK Reference Laboratory for examination of amniotic fluid phospholipids (Stevenson et al 1987). A reprint of the publication is appended. Briefly, it was possible, on one TLC plate, to determine the concentration of PG, by PAS staining and densitometry, and to calculate the ratio of PC to SPH (the lecithin/sphingomyelin or L/S ratio) by planimetry following visualisation by method 2. For planimetry, a simple rectangular figure was constructed about the PC

and SPH spots and the ratio of their areas calculated. Assessment of foetal lung maturity was possible, based on this information. The PAS stain clearly revealed quantities of 0.1 µg of PG and the colour was stable for several hours.

The Rf values of the lipid classes listed above are shown in Table 6.2. PS and PE gave discrete Rf values when run as separate standards, but tended to comigrate when they occurred in complex mixtures. Fig 6.1 shows a plot of the PG concentration against integrator counts and the method of quantitation using densitometry is described on page 102.

TABLE 6.2 Rf VALUES FOR LIPIDS SEPARATED BY SYSTEM 1

Lipid Class	Rf value
diPG	.92
PA	.86
PG	.71
PS	.48
PE	.44
PI	.34
PC	.19
SPH	.10
lyso-PC	.06

TLC system 2

A Whatman K5 20 x 20 cm silica gel plate (250 µ layer) was run in the same solvent system as system 1, under identical conditions. Visualization of separated components was by method 2. The Rf values for this system are shown in table 6.3.

This system has the disadvantage that diPG and PG co-migrate, as do PI and PS. PE, however, is clearly resolved, as were lyso-PC, PA, SPH and PC.

TABLE 6.3 Rf VALUES FOR LIPIDS SEPARATED BY SYSTEM 2

Lipid Class	Rf value
PE	.54
diPG + PG	.43
PA	.38
PC	.26
PI + PS	.22
SPH	.16
lyso-PC	.12

TLC system 3

A Whatman K5 plate, was employed in the solvent system chloroform:methanol:acetic acid:water (50:25:8:1 by volume). Chromatography was under saturated conditions. Visualisation of separated components was by method 2 and Rf values for this system are shown in table 6.4. PS and PI were clearly resolved.

TABLE 6.4 Rf VALUES FOR LIPIDS SEPARATED BY SYSTEM 3

Lipid Class	Rf value
PE	.92
diPG	.90
PA	.81
PG	.74
PS	.61
PI	.39
PC	.18
SPH	.09
lyso-PC	.07

TLC system 4, separation of neutral lipid classes

A two-stage, one-dimensional system was employed (Bitman, Wood and Ruth 1981) to separate the neutral lipid classes. Whatman K5 plates were developed in diethyl ether to remove the binder incorporated by the manufacturer. Plates were developed to 170 mm in the first stage in the solvent mixture chloroform:methanol:acetic acid (98:2:1 by volume). After air drying, the plate was developed in the same direction in the solvent mixture hexane:diethyl ether:acetic acid (94:6:0.2 by volume) to the top of the plate. Development in both stages was under saturated conditions. Visualization was by method 3. Table 6.5 shows the Rf values obtained by this method. The lipid classes listed were as follows - cholesterol ester was 50% cholesteryl oleate, 50% cholesteryl linoleate. Triglyceride was 50% triolein, 50% trilinolein. 1,3-diglyceride was 50% 1,3-diolein, 50% 1,3-dilinolein. 1,2-diglyceride was 50% 1,2-diolein, 50% 1,2-dilinolein. Cholesterol was > 99% as provided by Sigma. Free fatty acid was 50% oleic acid, 50% linoleic acid. (It was not necessary to use unsaturated fatty acids exclusively, as a good response was also noted with saturated species). Monoglyceride was 50% 2-monoolein, 50% 2-monolinolein. Phospholipid was 50% PC, 50% PE.

TABLE 6.5 R_f VALUES FOR LIPIDS SEPARATED BY SYSTEM 4

Lipid Class	RF value First Stage	Second Stage
Cholesteryl ester	.71	.80
Triglyceride	.71	.70
1,3-Diglyceride	.66	.62
1,2-Diglyceride	.60	.57
Cholesterol	.52	.50
Free fatty acid	.40	.39
Monoglyceride	.22	.22
Phospholipid	origin	origin

TLC system 5

A Whatman K5 plate was run in two dimensions. In the first the solvent system employed was chloroform:methanol:water (65:25:4 by volume). The chromatogram was developed 125 mm, then dried in a stream of nitrogen. Following drying, the plate was placed in the second solvent system - chloroform:methanol:acetone:acetic acid:water (65:10:20:10:3 by volume) and developed to 125 mm at 90^{o} to the first dimension. Both dimensions were run under saturated conditions.

This system resolved the lipid classes of the standard mixture. The R_f values are shown in table 6.6. This system was used to check the purity of individual lipid standards purchased from commercial houses, and to test the systems described above. Visualisation was by method 2.

TABLE 6.6 Rf VALUES FOR LIPID CLASSES SEPARATED BY SYSTEM 5

Lipid Class	Rf value 1st Dimension	2nd Dimension
diPG	.37	.51
PA	.34	.59
PG	.41	.43
PS	.26	.16
PE	.52	.41
PI	.23	.09
PC	.30	.19
SPH	.17	.06
lyso-PC	.11	.03

Quantitation

Quantitation was principally by scanning transmission densitometry on a Helena Laboratories Quick-Scan Jnr TLC instrument (MI Scientific, Newcastle upon Tyne).

The instrument comprised a tungsten lamp with facilities for filtering of wavelength, over the range 420-675 nm. A beam of light passed through a slit (0.2 mm x 2 mm) and through the developed chromatogram, which was moved across it by a motorised mechanism. Transmitted light was collected by a photomultiplier and amplified, the spots being translated into peaks on the instrument chart recorder. Also on the chart is an integrator read-out, enabling the "area under the curve" to be calculated.

After calibration of the instrument by use of a neutral density transparency, supplied by the manufacturer, the machine was ready for use. For each lipid class to be quantified, it was necessary to run a series of concentrations on each chromatogram. By scanning

these standards, a calibration curve could be constructed for each class of interest. This ensured that for each run a new calibration curve was prepared. See figure 6.2(a-i). A problem in densitometry is the difficulty in manually applying standard sized spots to the layer. A Desaga Autospotter was employed (Uniscience Ltd, Cambridge) to overcome this problem.

A further method of quantitation was also employed for PS in System 3. The plate was sprayed with ninhydrin and warmed, as described in visualisation method 4, as above. Aminophospholipids such as PS and PE react, giving a purple colour. The PS spots, which were elliptical in shape, were clearly visible by this means. A rectangular figure was constructed about the spot and its area calculated. By running a range of concentrations of a standard preparation of PS it was possible to construct a standard curve which was linear over a wide range (see fig 6.3).

Comment

There were two reasons for separating the lipid classes in the manner described above. Firstly, no single one-dimensional thin layer chromatographic system, in the authors hands, was able to resolve all the lipids of interest. Secondly, because of diffusion of separated spots, quantitation by densitometry was not possible in two-dimensional systems. This meant that a test sample had to be chromatographed in four separate systems. In each case appropriate standards were applied to the TLC plate so that a calibration curve could be constructed for each lipid class of interest (Fig 6.2a-i).

System 1 allowed the quantitation, by this means, of diPG, PG, PC and SPH. System 2 was used to separate and quantify PE, PA and lyso-PC. System 3 was able to separate and quantify PS and PI. The neutral lipids were separated and quantified by System 4.

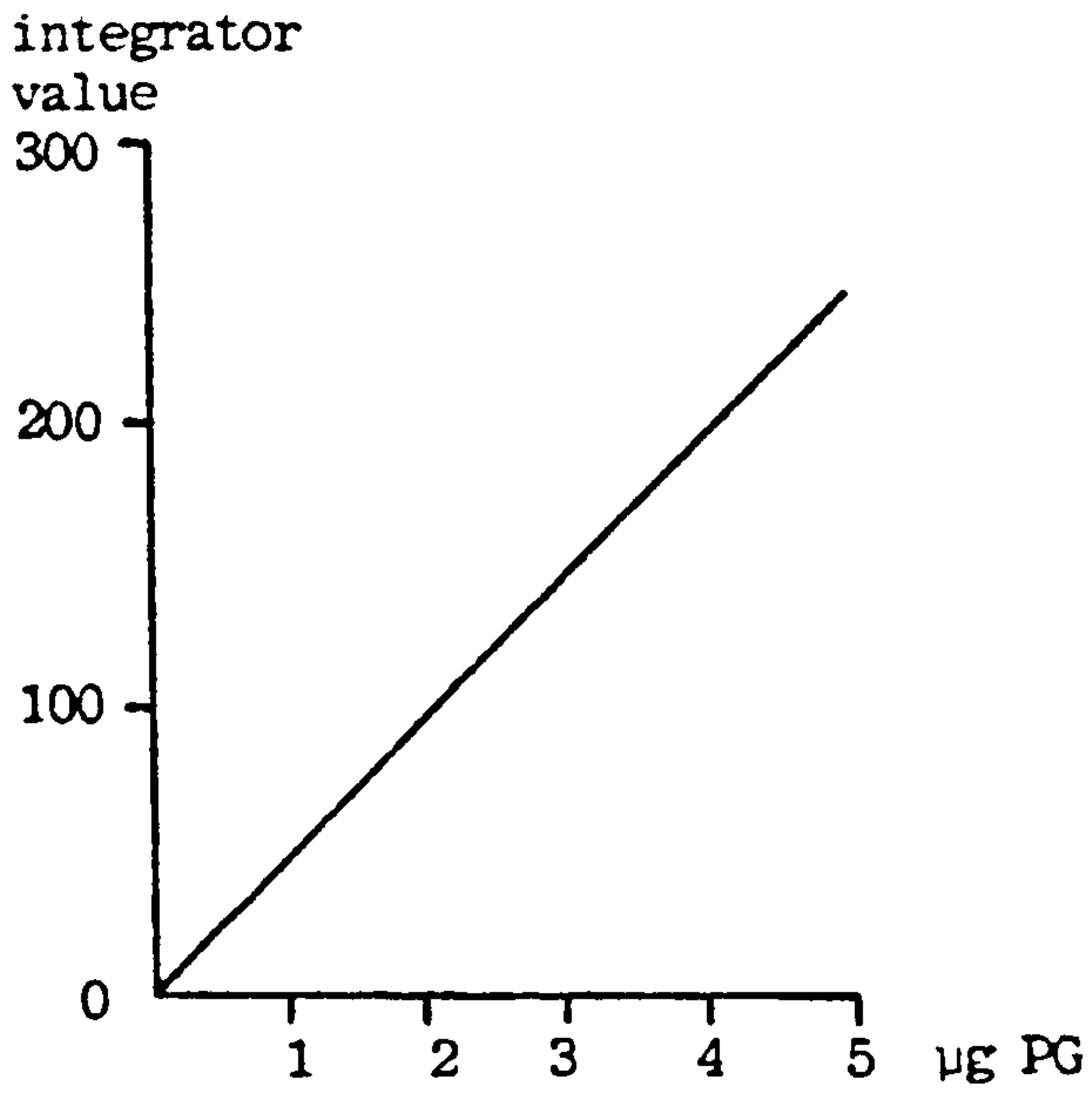


Fig. 6.1 standard curve of PG vs. integrator value

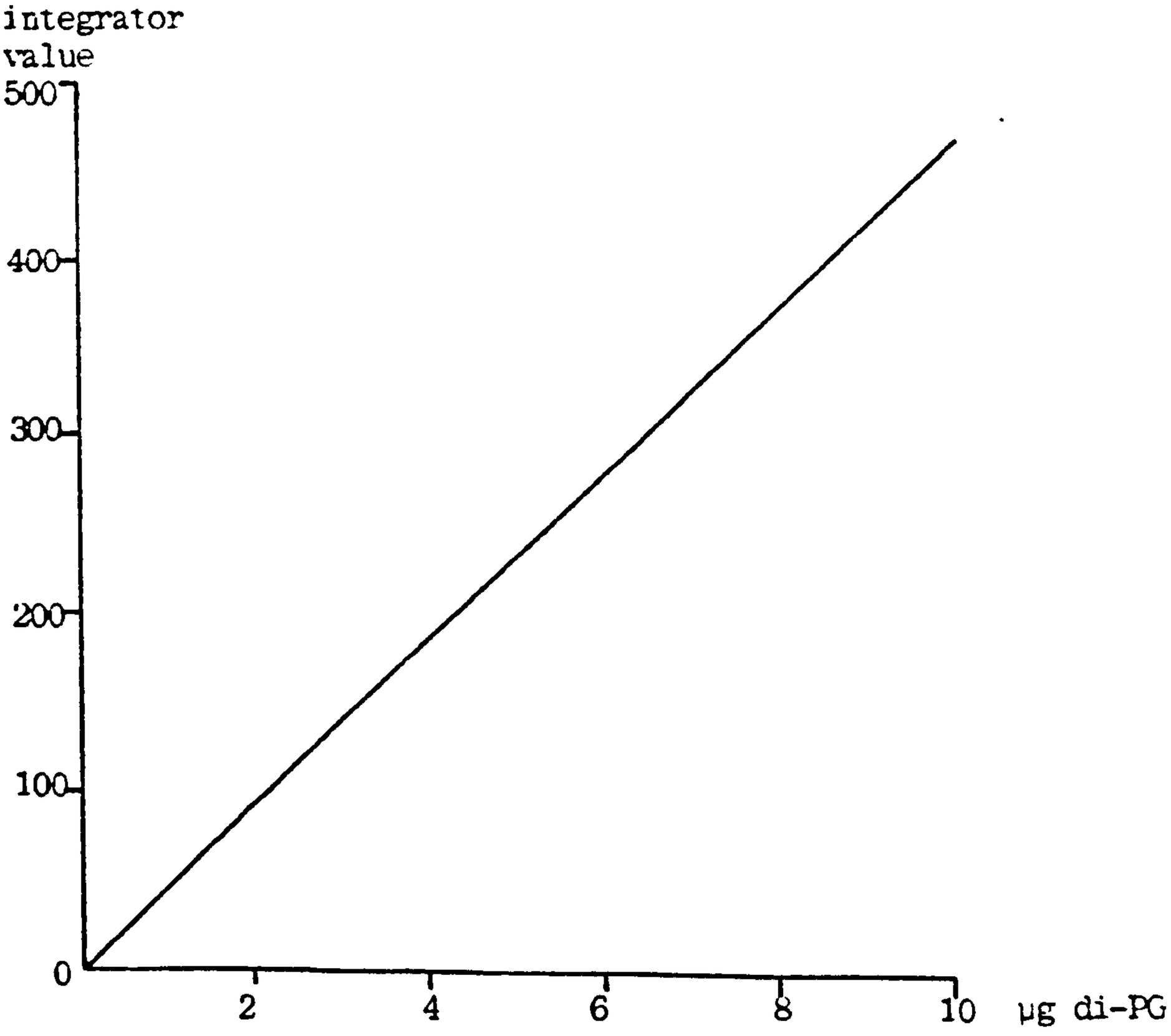


Fig 6.2a standard curve of di-PG vs. integrator value

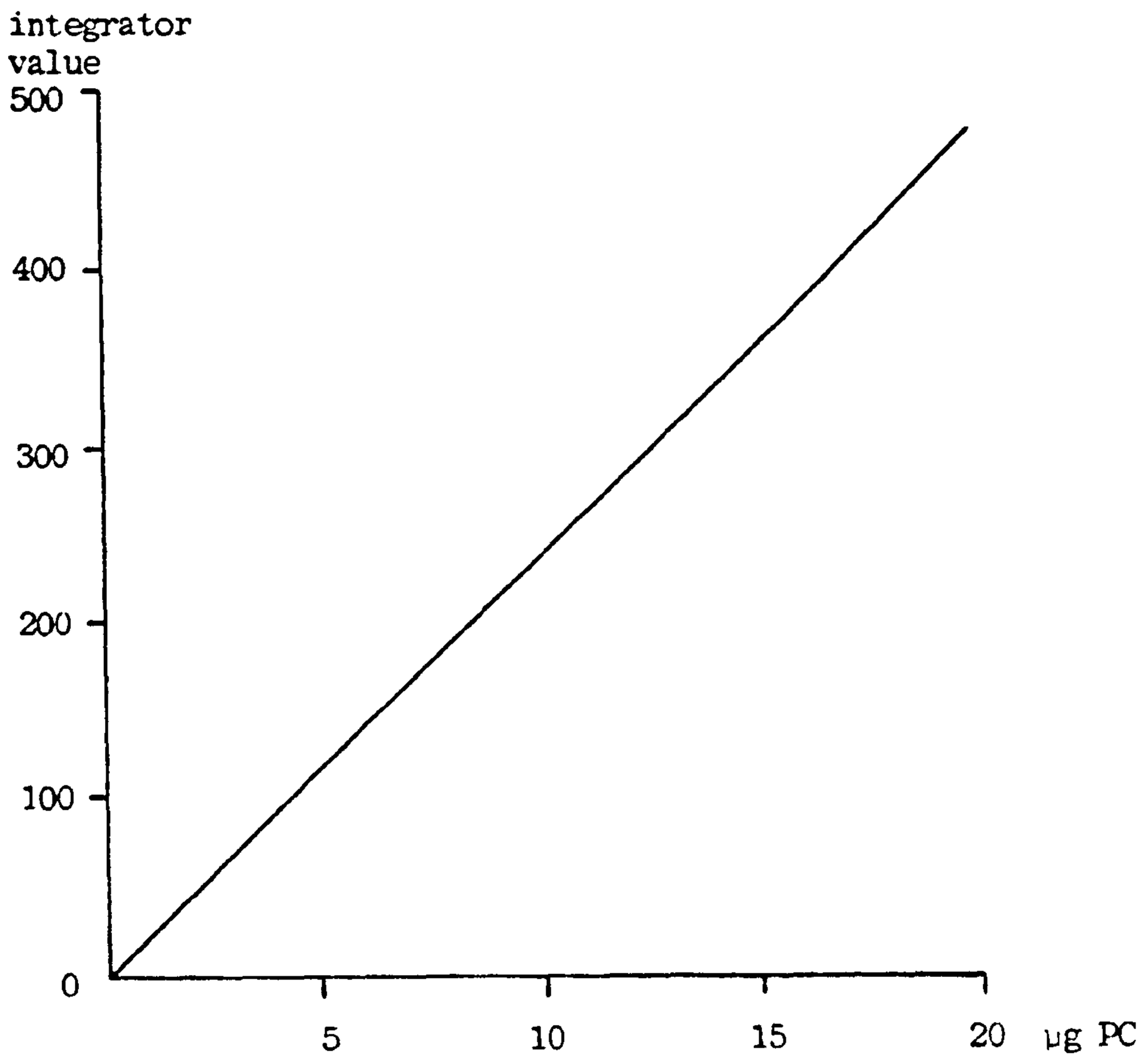


Fig 6.2b standard curve of PC vs. integrator value.

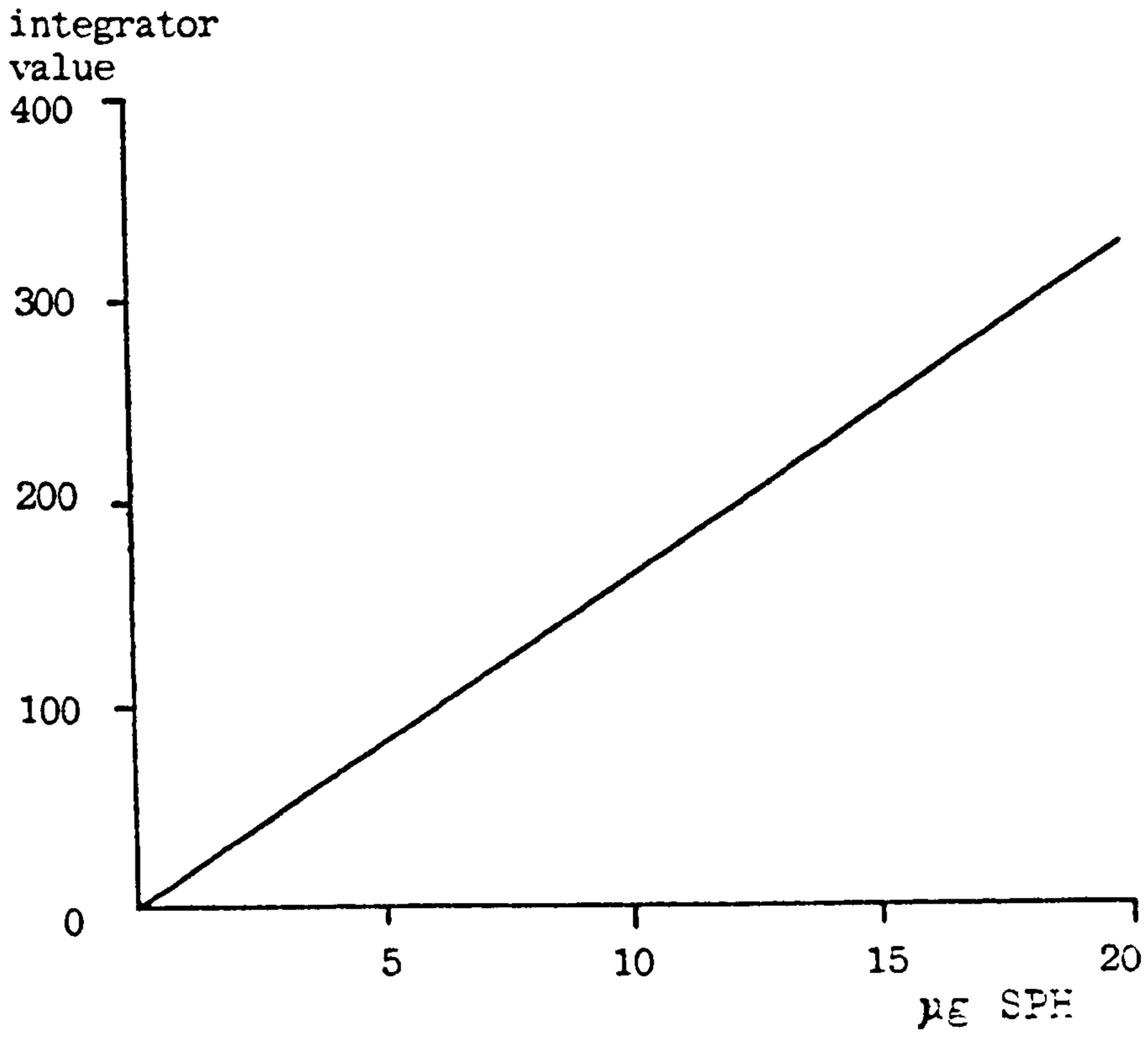


Fig 6.2c standard curve of SPH vs. integrator value.

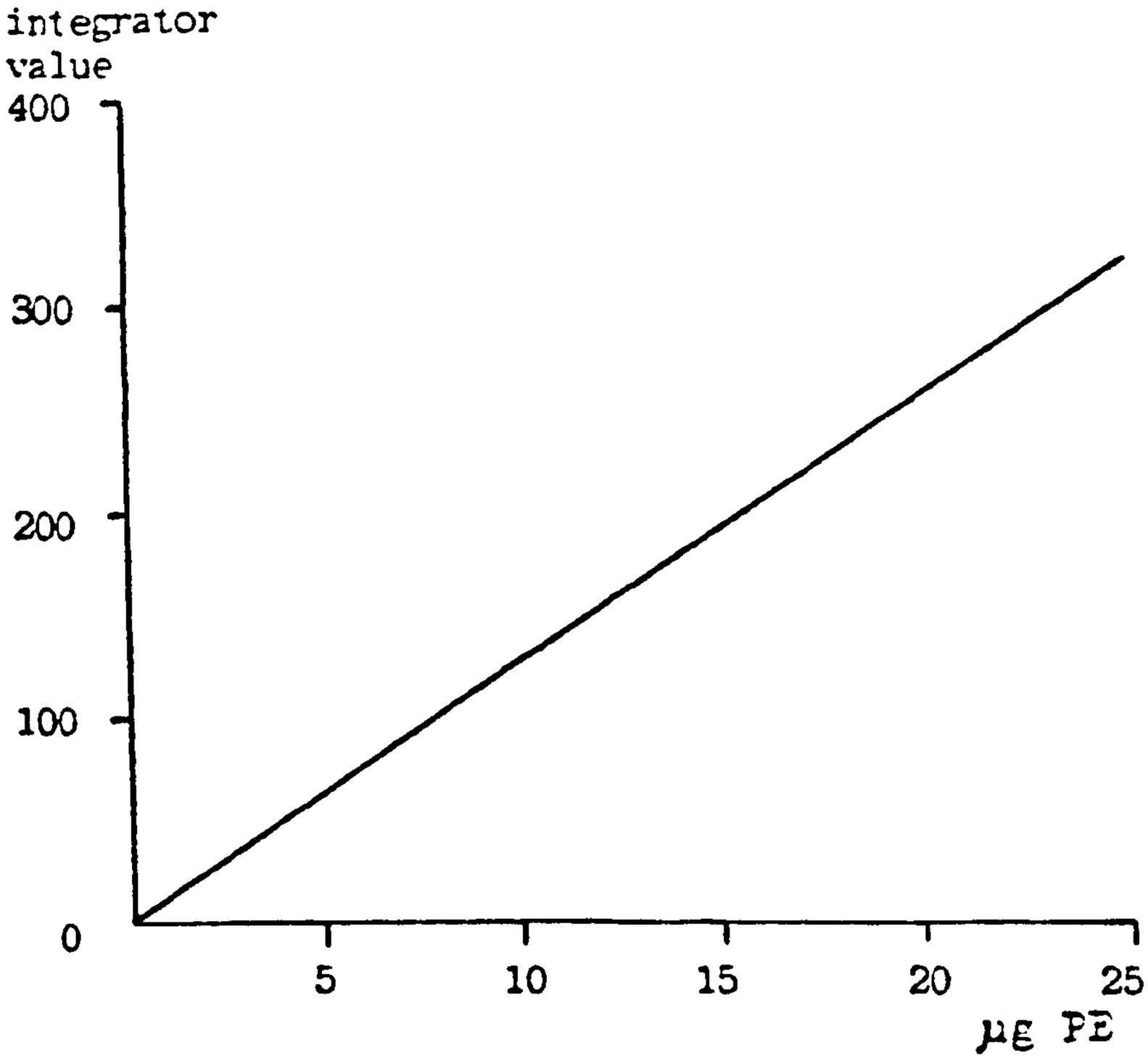


Fig.6.2d standard curve of PE vs. integrator value.

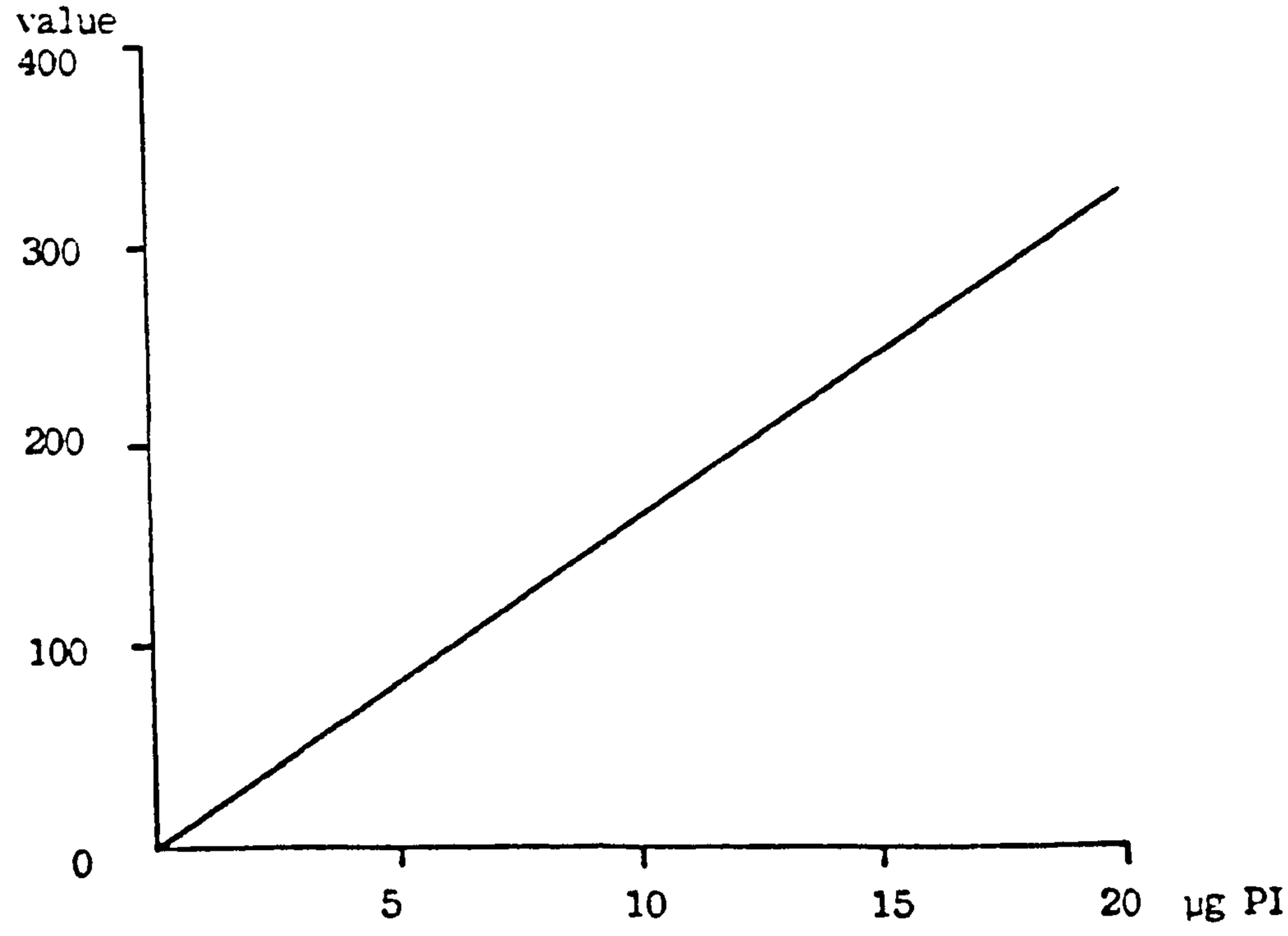


Fig.6.2e standard curve of PI vs. integrator value.

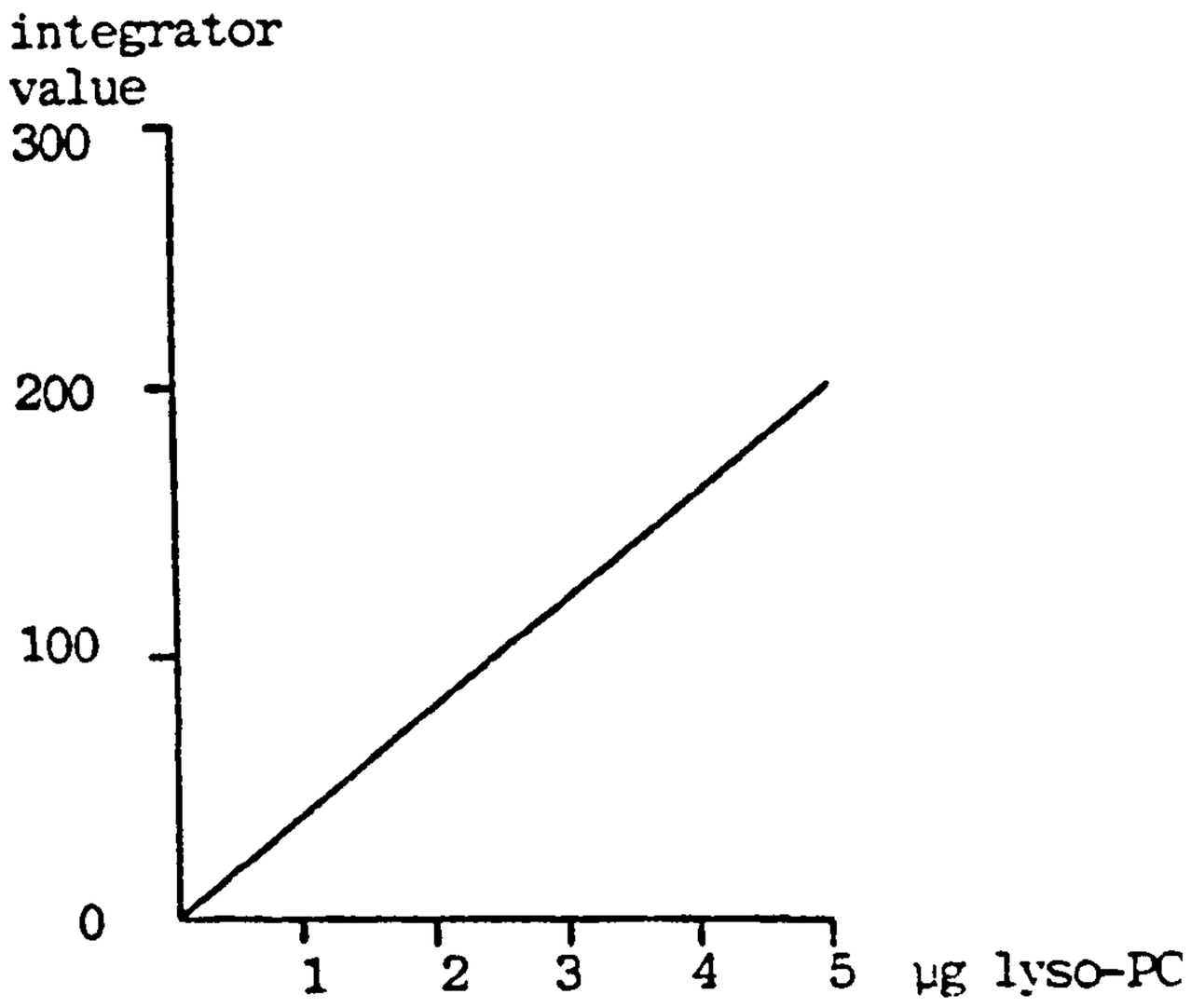


Fig.6.2f standard curve of lyso-PC vs. integrator value.

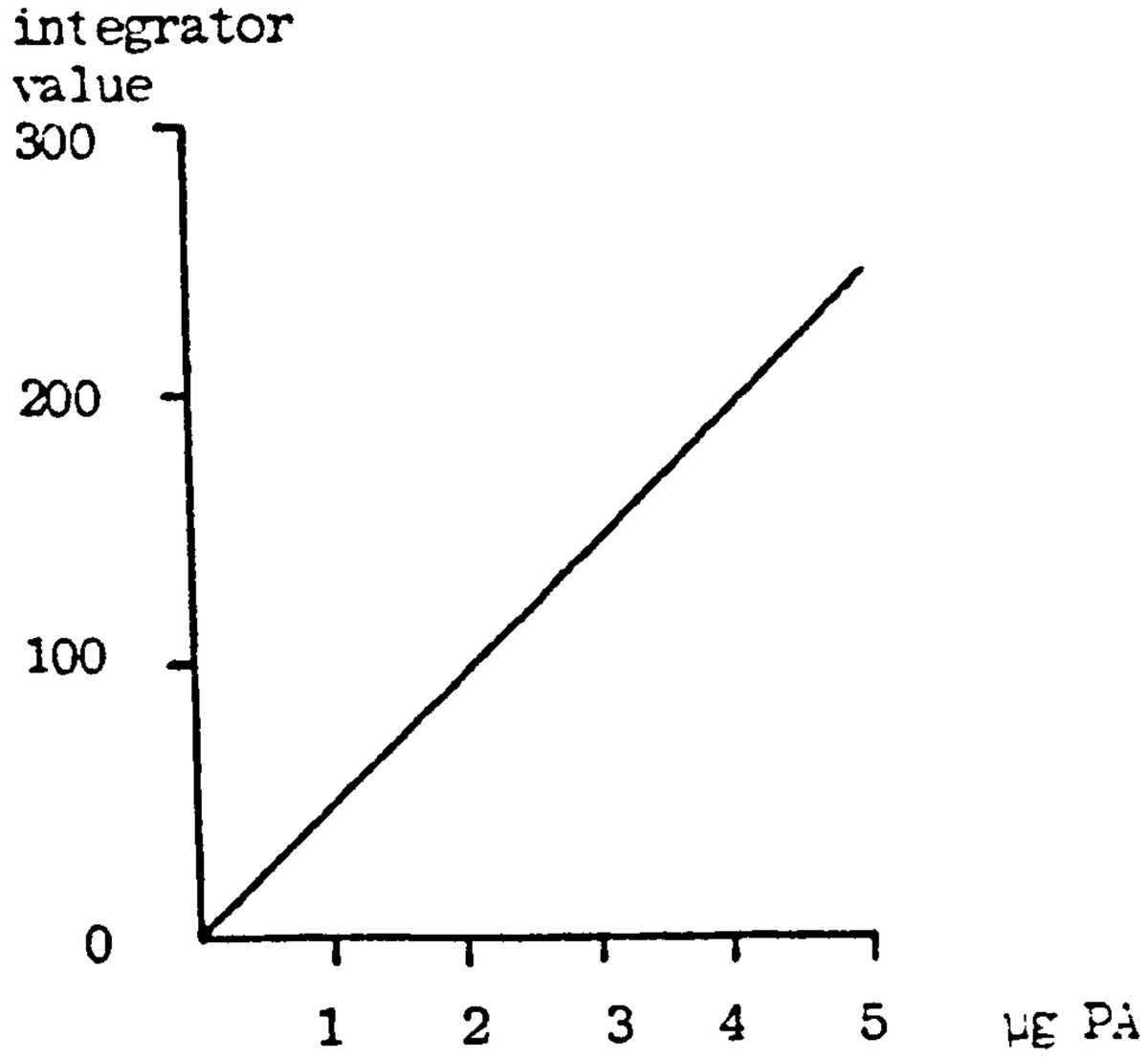


Fig.6.2g standard curve of PA vs. integrator value.

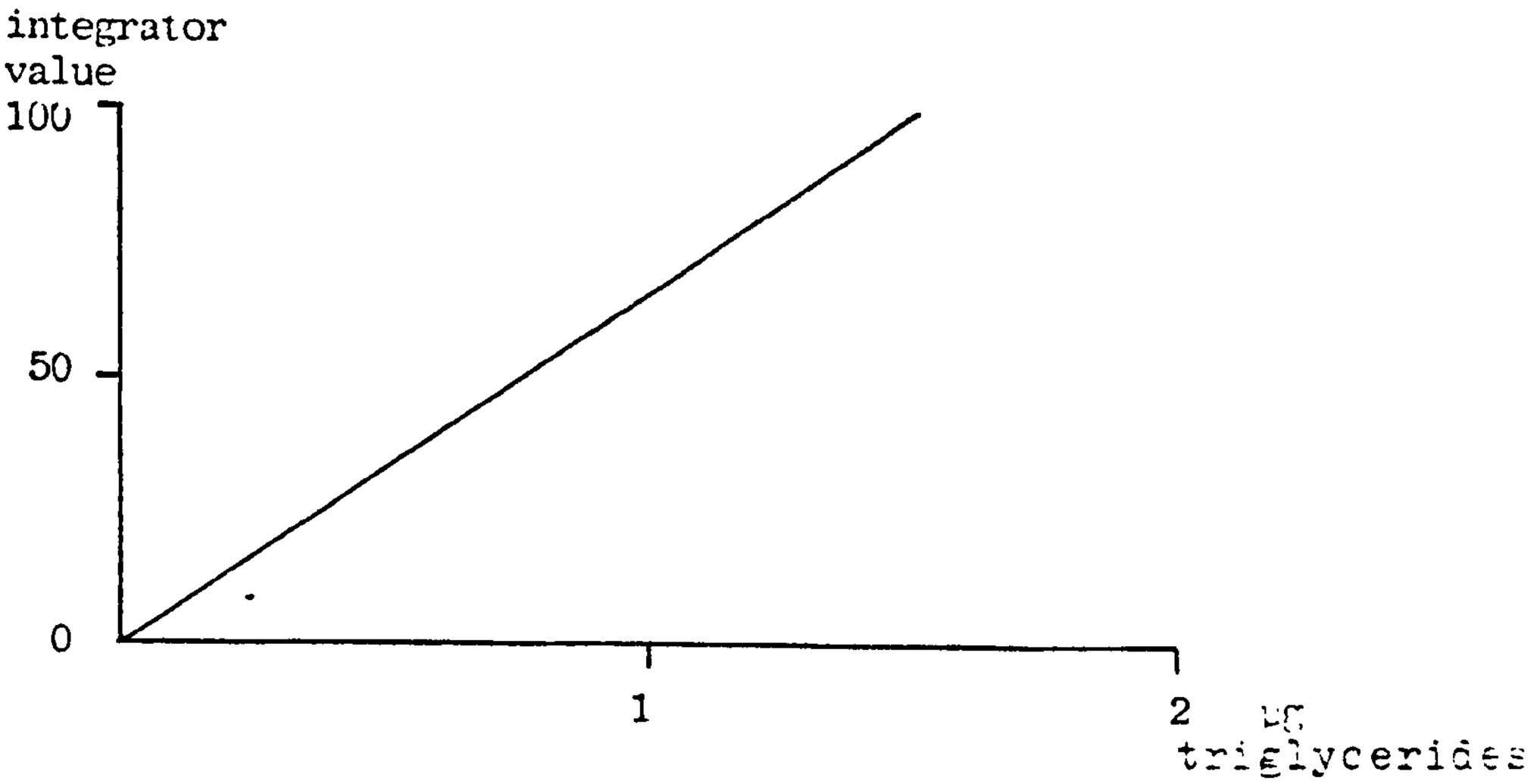


Fig 6.2h standard curve of triglycerides vs. integrator value.

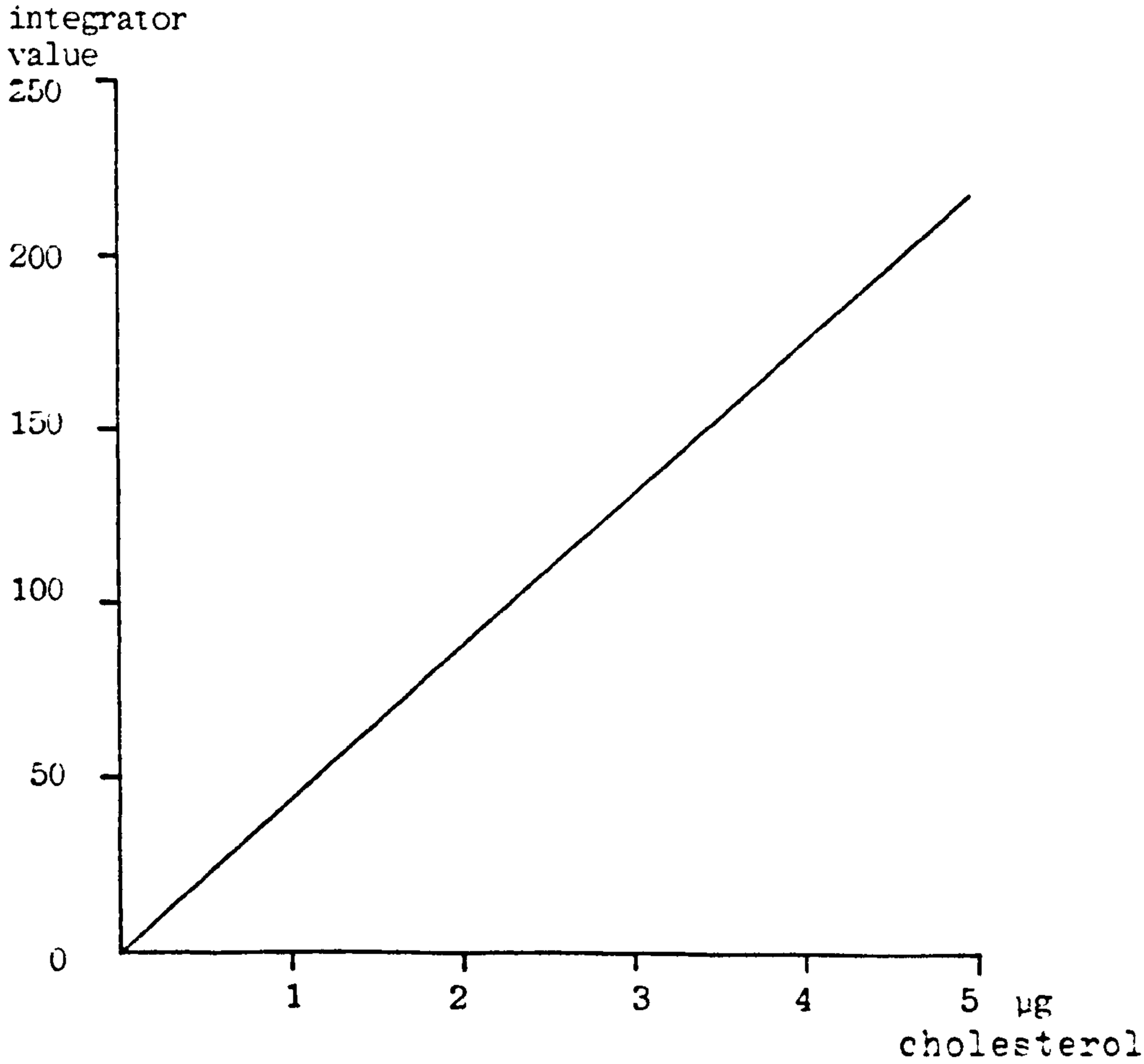


Fig 6.2i standard curve of cholesterol vs. integrator value.

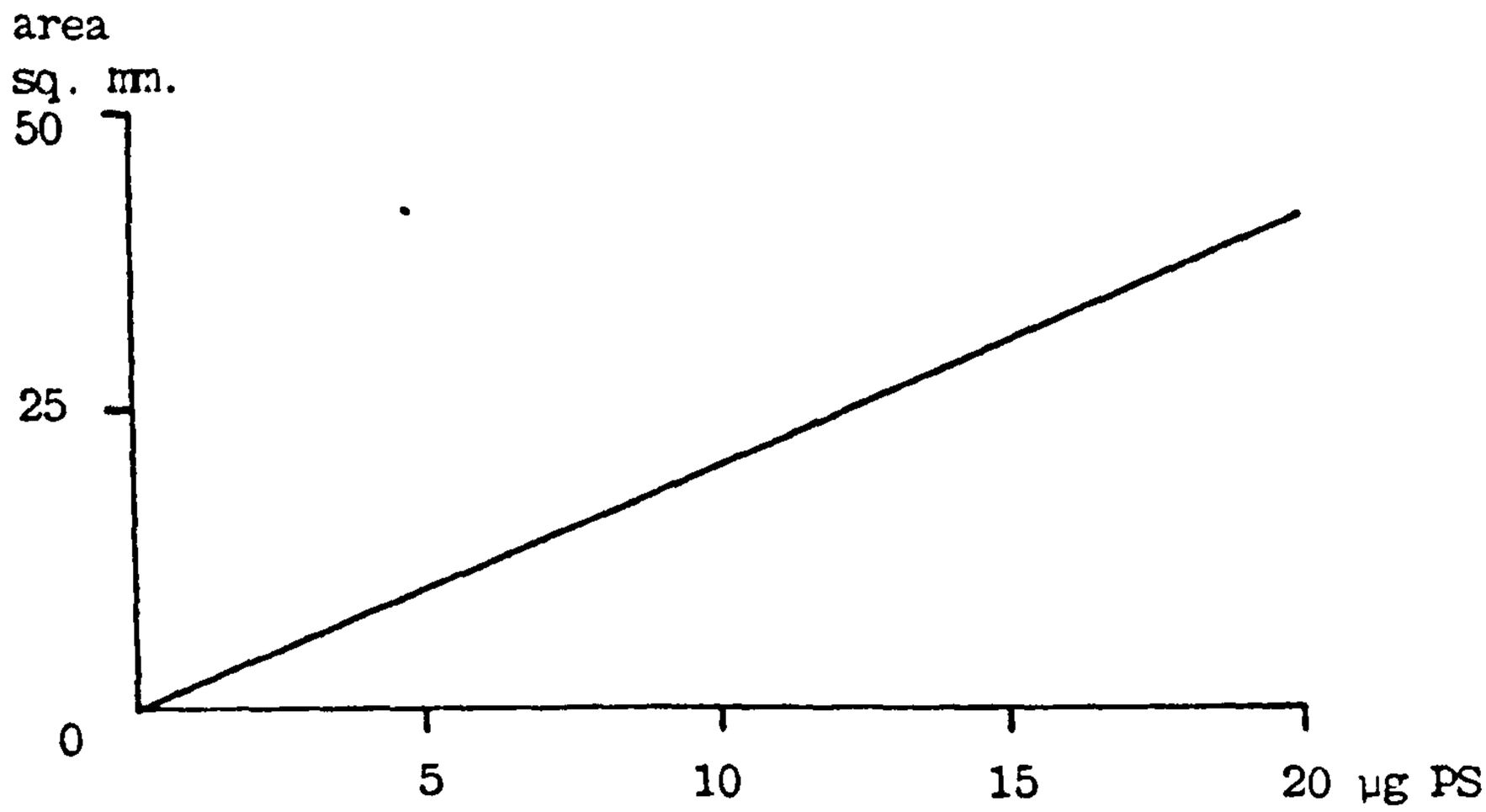


Fig. 6.3 standard curve of PS by.planimetry.

6.2 DETERMINATION OF PHOSPHOLIPID PHOSPHORUS

TLC plates were sprayed with 50% sulphuric acid in methanol and charred at 130°C. Phospholipid spots were marked by drawing around them with a fine pencil. Exposing the surface of the plate to UV irradiation at 355 nm made this easier since the spots showed some fluorescence. An aliquot of lipid mixture under test was then applied to the plate in order to provide "total" value of phospholipid phosphorus. Digestion and colour development were as described by Rouser et al (1966).

Spots were then scraped from the plate into pyrex tubes (150 x 18 mm). An equal area of silica gel was removed to provide a "blank" value. 0.9 cm^3 of 72% perchloric acid was added to each tube and digestion of the spots was carried out for 40 minutes at 150°C in an electrically heated apparatus, in a fume cupboard. The fume cupboard was built with this function in mind, since there was no exposed wood internally, (surfaces being resin treated) and its base was made of ceramic. The fume extract duct was fitted with a scrubbing device.

Following digestion, the walls of the tubes were washed with 5 cm^3 of distilled water and 1 cm^3 of 22 mmol dm^{-3} ammonium molybdate was added and mixed followed by 1 cm^3 of 0.57 mol dm^{-3} ascorbic acid and 2 cm^3 of distilled water. The mixture was boiled for 10 minutes in the electrically heated block set at 120°C. A reagent blank was carried through the process.

After colour development the tube contents were centrifuged to settle particulate material and absorbance read at 820 mm. A standard curve was prepared using KH_2PO_4 (1.3613 g dm^{-3} is equivalent to 310 µg cm^{-3} of phosphorus) in the range up to 100 µg of phosphorus. A standard curve is shown in fig 6.4.

Glassware employed in this assay was scrupulously cleaned using phosphate-free detergent (Extran 300, BDH, Poole).

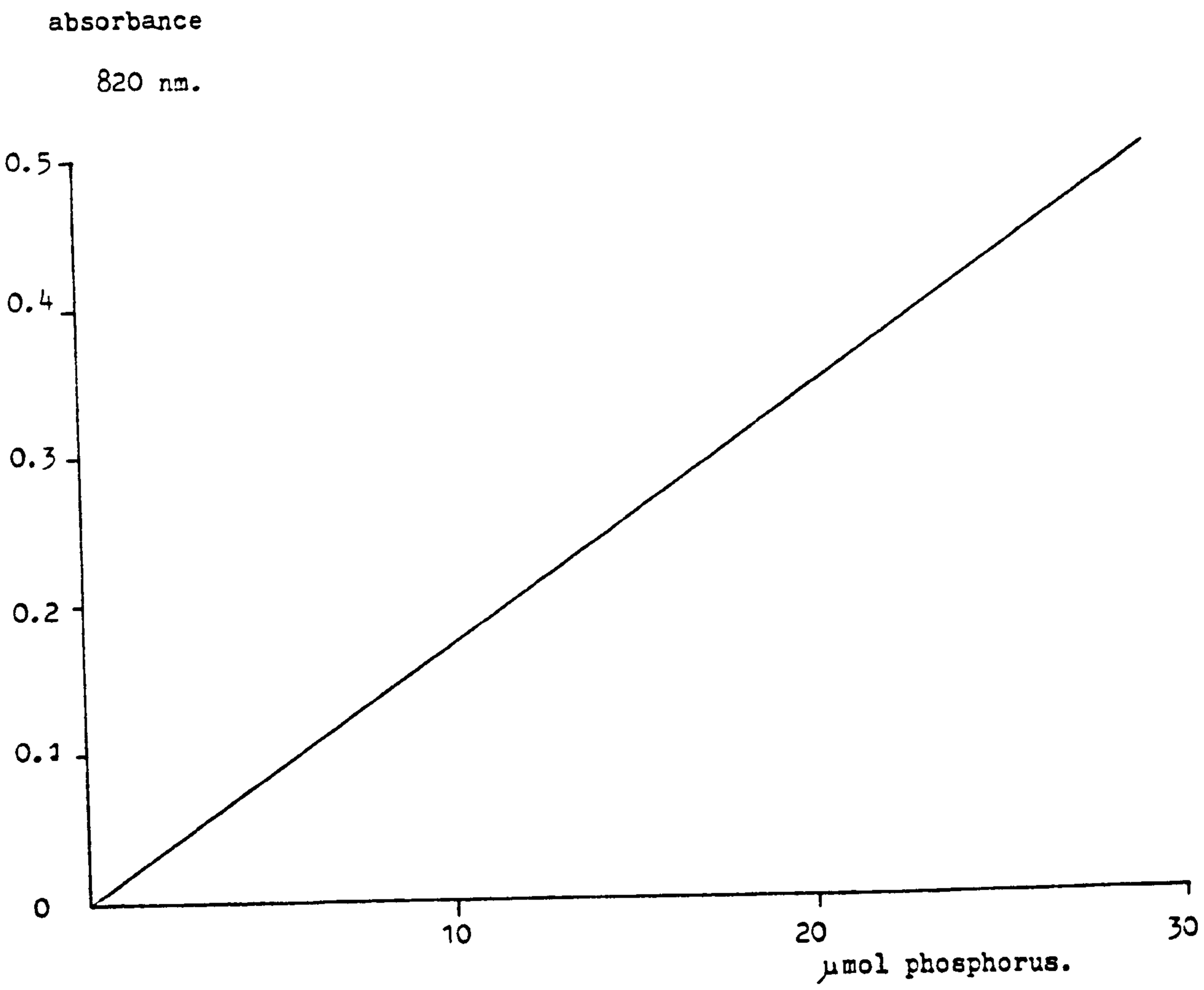


Fig 6.4 Standard curve of phosphorus concentration vs. absorbance

6.3 DETERMINATION OF PHOSPHOLIPIDS BY REACTION WITH AMMONIUM FERROTHIOCYANATE

The method is based on the formation of a coloured complex between ammonium ferrothiocyanate and phospholipids which is soluble in chloroform (Stewart 1980).

Phospholipids were dissolved in 2 cm^3 of chloroform, added to 2 cm^3 of ammonium ferrothiocyanate (see below) and vigourously mixed on a vortex mixer for 1 minute. On separation, the lower chloroform phase was removed using a Pasteur pipette, clarified with a pinch of anhydrous sodium sulphate, and the absorbance read at 488 nm.

Calibration curves were prepared for a range of phospholipids and are shown in figure 6.5. The variation in slope of the curves presumably results from the formation of a range of complexes. Stewart (1980) believed that lyso-PC and SPH probably form 1:1 complexes with $Fe(SCN)_3$ while PE and PS probably form 2:1 complexes.

Ammonium ferrothiocyanate solution was prepared by dissolving 27.03 g (0.1 mol) of ferric chloride hexahydrate ($FeCl_3.6H_2O$) and 30.4 g (0.4 mol) of ammonium thiocyanate (NH_4SCN) in deionized water and making up to 1 dm^3. The solution is stable for at least 6 months at room temperature.

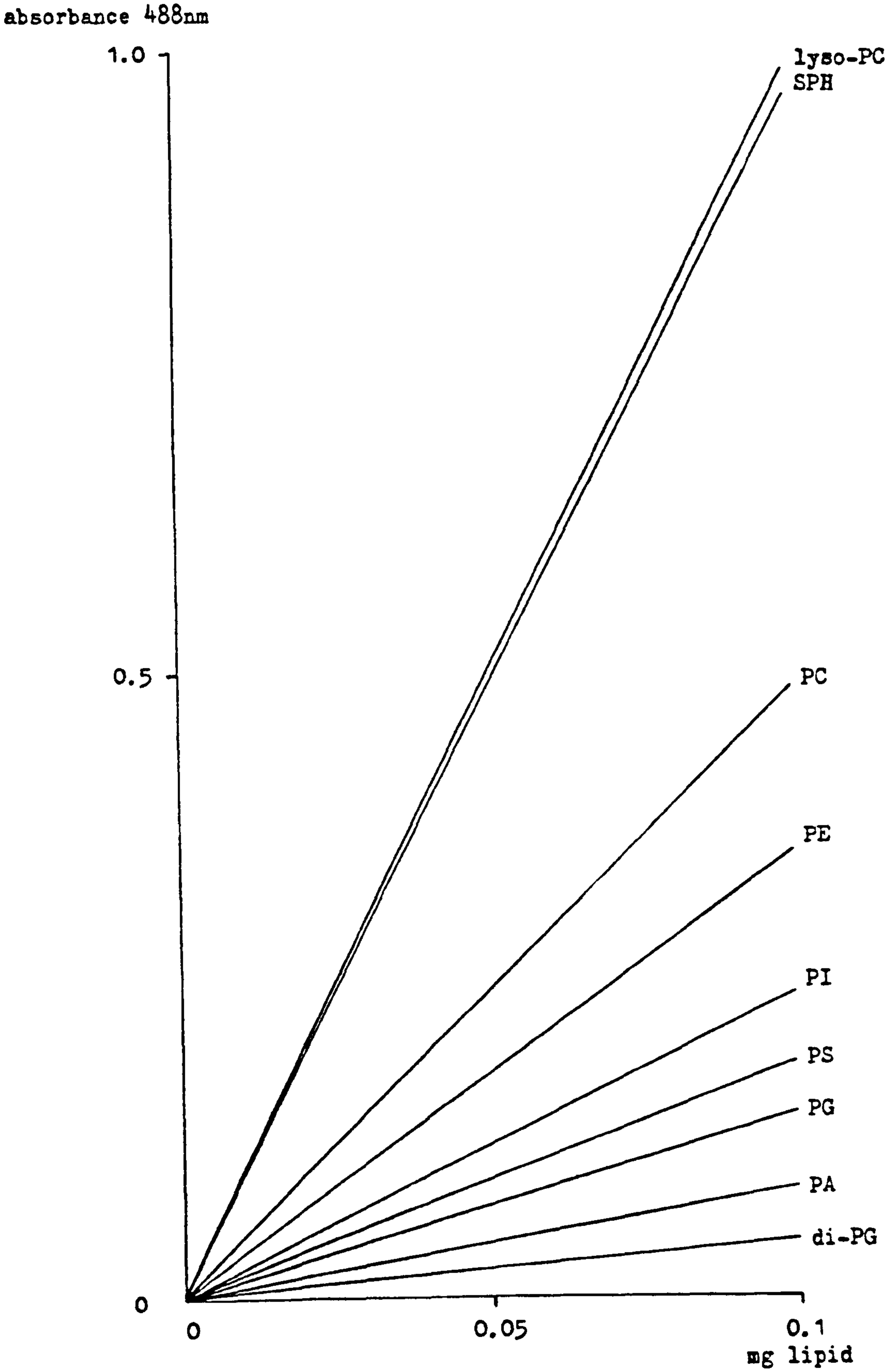


Fig 6.5 standard curves for phospholipid phosphorus estimation.

6.4 ESTIMATION OF FATTY ACIDS BY GAS LIQUID CHROMATOGRAPHY OF THEIR METHYL ESTERS ON CAPILLARY COLUMNS

Apparatus - the gas chromatograph employed was a Pye Unicam GCD, fitted with flame ionizer detectors. This instrument was designed for use with packed columns and was originally fitted with dual injection ports, columns and detectors. The fan assisted oven was controllable over the temperature range from ambient up to 400°C and programming of temperature was possible. Flame ionizer detector (FID) temperature and injector temperature were adjustable over the same range.

A number of modifications were undertaken in order to convert the instrument to capillary column use. The capillary columns and all the materials required for this conversion were obtained from Phase Separations, Queensferry.

Firstly, an "on-column" injector was fitted. This device, manufactured by Scientific Glass Engineering, Milton Keynes (model no OCI-3) enabled the injection of 1 µl samples directly on to the capillary column. A 5 µl syringe was used, fitted with a stainless steel sheathed vitreous silica needle. The sheath extended to 20 mm from the end of the needle, so that the needle was free to penetrate into the capillary column. The injector device was cooled by a stream of compressed air flowing at 300 cm^3 min^{-1}, controlled by a needle valve. Cooling in this way prevented heat build up from the chromatograph oven, which could have volatilized the lower boiling components of the samples under test. The major advantage

of the "on column" system was that the entire sample was placed directly into the column and there were no losses, as might have occurred in a sample splitting system. While sample injection was being carried out, a pneumatically operated seal gripped tightly around the needle sheath and continued to keep the system sealed when the needle was withdrawn.

Hydrogen was the preferred carrier gas. It was necessary to fit an accurate regulator valve to control the H_2 supply to the injector and column-a regulator calibrated in the range 0-1 atmosphere was found to be adequate. The FID was originally designed to accommodate carrier gas flows of 30-60 cm^3 min^{-1}. Since the flow of hydrogen from a capillary column was around 2 cm^3 min^{-1}, detector sensitivity was drastically reduced and an auxilliary supply of "make-up" gas had to be provided. Nitrogen was the gas of choice and was connected, via a T-piece, between the end of the capillary column and the FID. The pipework carrying the nitrogen make-up was led through the column oven so that the gas was heated to column temperature. This was to ensure that no condensation of high boiling components took place at the end of the column.

Gases were obtained from the British Oxygen Company and were industrial grade in the case of compressed air and oxygen-free grades in the case of nitrogen and hydrogen. Molecular sieves were employed in the gas lines and particle filters were also fitted to trap any entrained dust from the molecular sieves. Molecular sieves were regenerated by heating at 350°C for 24 hours.

While it was arguable that gas driers were not absolutely essential when using silicone gum phases like OV1, dryness was more important when the highly polar phase OV351 was used. Prolonged column life justified such precautions. Further, highly polar phases were susceptible to oxidation. No problems were encountered, however, when oxygen-free grade hydrogen was employed as carrier gas. Hydrogen was used as the carrier because of its high efficiency (Jennings 1980). The decision to use hydrogen raised a number of important questions. Firstly there was the problem of the possibility of interaction between the carrier gas and sample components or the liquid phase. Grob (1979) addressed this problem and concluded that in the absence of a suitable catalyst this was unlikely. The other hazard in the use of hydrogen was its capacity to form explosive mixtures with air over a wide range of concentrations. It may be argued that because of its high diffusivity it would be difficult to accumulate enough hydrogen to form an explosive mixture, even inside a chromatograph oven. Happily, reports of explosions in gas chromatographs are rare but it nevertheless remained prudent to take every precaution.

Gas cylinders, including hydrogen, were stored in a purpose built outhouse and connected to the laboratory by metal pipework laid underground. Inside the laboratory, the gas pipelines terminated on the bench at a set of colour coded screw taps. This work was carried out by Medical Gas Installations, Bolton. Molecular sieves and filters were connected to these and the chromatograph plumbed in using 1/8 inch outside diameter copper pipe. A strict routine of checking for leaks was maintained, both

in the chromatograph and in the gas supply lines inside the laboratory.

The Pye Unicam GCD was of an old design, originally comprising two injectors and two FIDs. There was also a 1/4 inch diameter hole in the roof of the oven for inserting a thermometer. Since one injector and one FID were redundant this meant that two more ports were opened in the oven roof. The oven door was hinged at the bottom and secured in place by two magnetic catches. These features meant that any leak of hydrogen would have been unlikely to reach a critical level in the oven and in the event of an explosion the door would have burst open more readily than would be the case with some modern instruments which have very tight fitting and securely latched doors.

Two columns were employed. These were flexible silica columns ("Flexsil", Phase Separations, Queensferry) wall coated with OV1 and OV351 respectively, i.e., a non-polar phase and a highly polar phase. Each measured 25 metre x 0.32 mm i.d. with a wall-coating 0.15 microns thick. When using the OV1 column, temperature was programmed to maintain 130°C for 2 minutes, then increased to 300°C at 4°C/min. Detector temperature was 275°C. The maximum temperature limit for OV1 was 300°C.

When the OV351 column was in use, temperature was programmed to maintain 130°C for 2 minutes then increased at 4°C/min, to 240°C. Detector temperature was 275°C. The maximum temperature limit for OV351 was 250°C.

Test mixtures, obtained from Phase Separations, were applied periodically to each column in order to check performance. Tables 6.7 and 6.8 illustrate the order of elution of their components.

TABLE 6.7 TEST MIXTURE ON OV 1

order of elution	component
1	nonane
2	decane
3	1-octanol
4	2,6-dimethylphenol
5	undecane
6	cis, trans-2-propylcyclohexanol
7	2,6-dimethylaniline
8	napthalene
9	dodecane
10	tridecane

TABLE 6.8 TEST MIXTURE ON OV 351

order of elution	component
1	decane
2	undecane
3	dodecane
4	tridecane
5	1-octanol
6	cis, trans-2-propylcyclohexanol
7	napthalene
8	2,6-dimethylaniline
9	2,6-dimethylphenol

It was necessary to calculate, for each column, the response factor (RF) for each fatty acid methyl ester and its retention time relative to the internal standard. The methyl ester of heptadecanoic acid was selected as internal standard. Two

concentrations of each FAME, 0.1 µg and 0.25 µg, were run on each column. Table 6.9 and 6.10 show the results obtained. This information was loaded into the Spectra Physics model SP4100 computing integrator which was connected to the gas chromatograph. The integration program (occupying some 32Kbytes of ROM in the SP4100) was able to identify each component by its relative retention time and to quantify each component by its response factor value. A further 6.5Kbytes of RAM was available on the SP4100 which was sufficient for operating some small statistical programs (see Chapter 6.21). Programs were stored on cassette tapes.

TABLE 6.9 SUMMARY OF CHROMATOGRAPHIC DATA. OV 1

FAME	RT	RRT	RF
10:0	3.38	0.192	0.750
12:0	6.32	0.359	0.541
14:1	10.16	0.577	0.395
14:0	10.63	0.604	0.472
16:1	14.71	0.836	0.396
16:0	15.24	0.866	0.407
17:0	17.60	1.000	-
18:3ω6,9,12	18.50	1.051	0.572
18:3ω9,12,15	18.99	1.079	0.470
18:2	19.02	1.081	0.406
18:1	19.24	1.093	0.492
18:0	19.84	1.127	0.537
20:4	22.37	1.271	0.651
20:2	23.28	1.323	0.493
20:3	23.43	1.331	0.564
20:1	23.55	1.338	0.469
20:0	24.16	1.373	0.732
22:6	26.21	1.489	0.527
22:2	27.05	1.537	0.411
22:1	27.68	1.573	0.431
22:0	28.14	1.599	0.472
24:1	31.22	1.774	0.418
24:0	31.89	1.812	0.495
26:0	35.29	2.005	0.493

RT = retention time
RRT = relative retention time
RF = response factor

TABLE 6.10 SUMMARY OF CHROMATOGRAPHIC DATA. OV 351

FAME	RT	RRT	RF
10:0	4.57	0.244	0.756
12:0	7.75	0.414	0.567
14:0	11.98	0.636	0.474
14:1	12.73	0.678	0.408
16:0	16.42	0.874	0.404
16:1ω9trans	17.00	0.905	0.430
16:1ω9cis	16.94	0.906	0.358
17:0	18.79	1.000	-
18:0	20.90	1.114	0.538
18:1ω9cis	21.44	1.137	0.546
18:1ω9trans	21.46	1.140	0.397
18:1ω11cis	21.55	1.145	0.531
18:2ω9,12cis	22.32	1.193	0.281
18:2ω9,12trans	22.35	1.199	0.527
18:3ω6,9,12cis	22.92	1.225	0.559
18:3ω9,12,15cis	23.85	1.265	0.481
20:0	25.33	1.341	0.736
20:1ω5cis	25.63	1.357	0.388
20:1ω11cis	25.56	1.366	0.553
20:2	26.64	1.414	0.496
20:4	27.52	1.475	0.661
20:3	27.96	1.483	0.586
22:0	29.47	1.557	0.504
22:1ω13cis	29.75	1.585	0.434
22:1ω13trans	29.77	1.593	0.426
22:2	30.16	1.598	0.406
24:0	34.05	1.803	0.493
22:6	34.04	1.820	0.578
24:1	34.41	1.832	0.413
26:0	40.37	2.152	0.497

RT = retention time
RRT = relative retention time
RF = response factor

Preparation of methyl esters of fatty acids (Metcalfe and Schmitz 1961)

14% boron trifluoride in methanol was obtained from Phase Separations, Queensferry. Material for esterification, freeze-dried or scraped from thin-layer chromatography plates, was placed in a pear shaped flask (Quickfit FP50/2). A few glass beads were added and 2 cm^3 of benzene pipetted into the flask to dissolve the material. 2 cm^3 of BF_3 in methanol was then added. The flask was connected to a condenser (Davies type, Quickfit C5/22) and the contents refluxed for 60 minutes in a boiling water bath. The flask was then removed and the reaction stopped by the addition of 1 cm^3 of water. Centrifugation at 500 x g separated the phases. The top layer, consisting of the methyl esters dissolved in benzene, was removed by pipette and stored in a tightly stoppered glass vial at $-40^{o}C$ until required.

Sample preparation for gas chromatography

Fatty acid methyl esters were stored in benzene at $-40^{o}C$. For chromatography, benzene was evaporated under a stream of dry nitrogen gas and the residue redissolved with 50 μl of octane. To this was added 50 μl of a 1 g dm^{-3} solution of the methyl ester of heptadecanoic acid, as internal standard. 1 μl of this mixture was injected via an on-column injector. Figures 6.6 and 6.7 show the chromatography, on OV1 and OV351, of FAMES of an APTT reagent, batch 117. Tables 6.11 and 6.12 show the identity of the arrowed peaks in each of the figures.

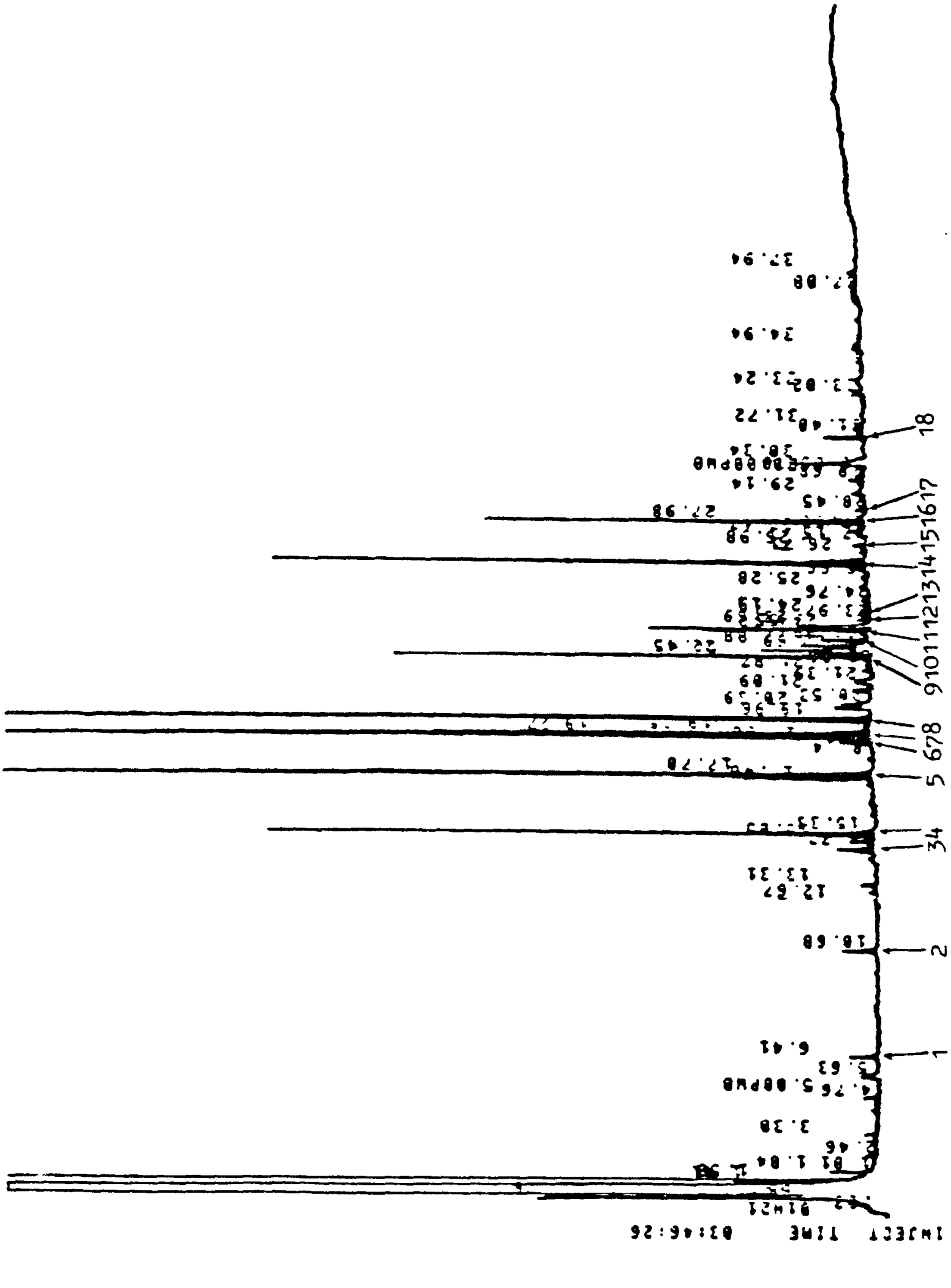


Fig. 6.6 FAMEs of 117 separated on OV 1

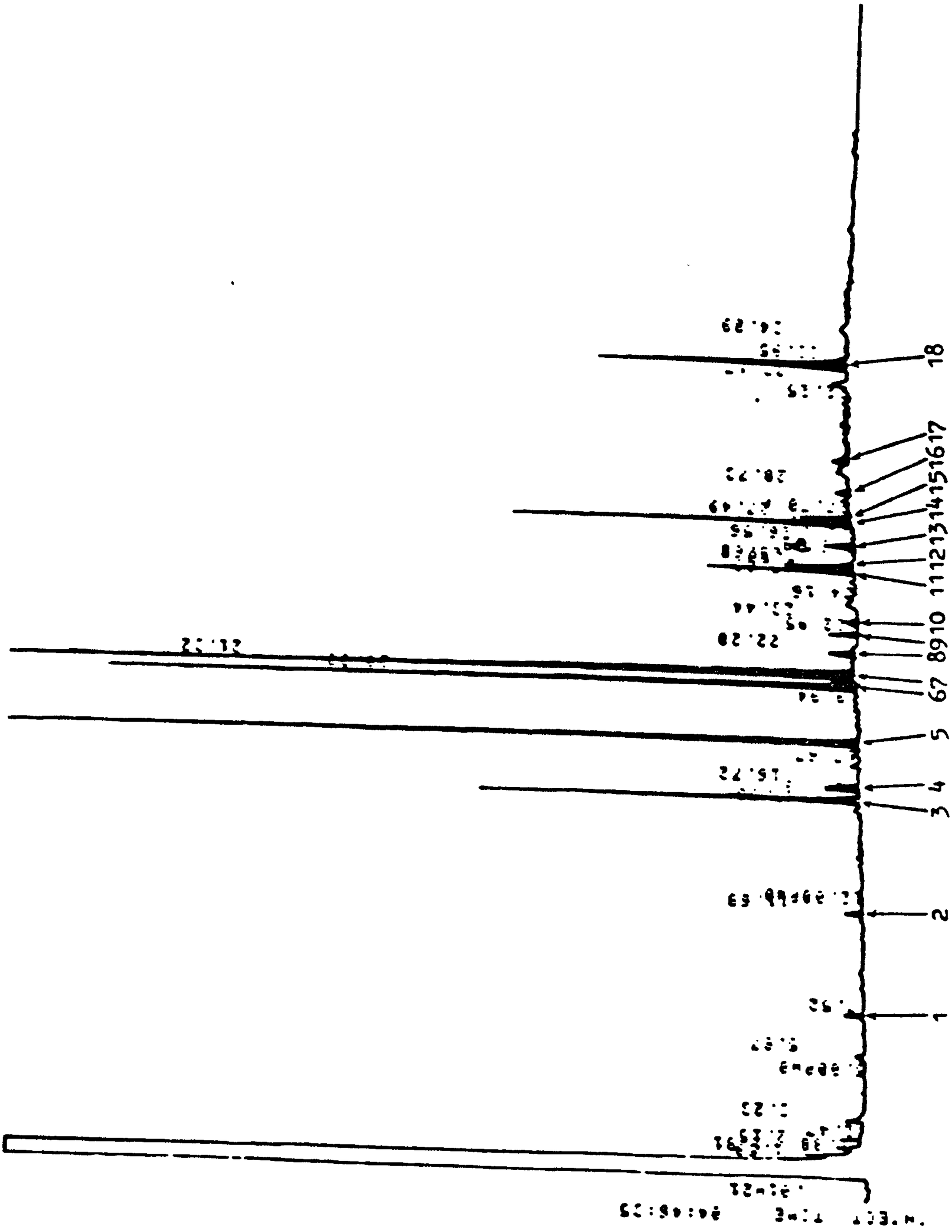


Fig. 6.7 FAMEs of 117 separated on OV 351

TABLE 6.11 IDENTITY OF PEAKS IN FIG. 6.6

Peak no.	FAME
1	12:0
2	14:0
3	16:1
4	16:0
5	internal standard (17:0)
6	18:3 ω 9,12
7	18:1
8	18:0
9	20:4
10	20:2
11	20:3
12	20:1
13	20:0
14	22:6
15	22:2
16	22:1
17	22:0
18	24:1

TABLE 6.12 IDENTITY OF PEAKS IN FIG. 6.7

Peak no.	FAME
1	12:0
2	14:0
3	16:0
4	16:1
5	internal standard (17:0)
6	18:0
7	18:1
8	18:2
9	18:3ω6,9
10	18:3ω9,12
11	20:0
12	20:1
13	20:2
14	20:4
15	20:3
16	22:0
17	22:1
18	22:6

6.5 TESTING OF PURITY OF PHOSPHOLIPID STANDARDS

Each phospholipid, before use as a chromatography standard or as a liposome component, was checked for purity. This was done by running it in the two-dimensional TLC system described earlier (system 5). A single spot was taken to indicate purity and preparations were discarded if other spots e.g. lyso-phospholipids or fatty acids were seen.

A further check was made on phospholipids used in manufacture of liposomes. These were examined by gas chromatography as described above (Ch 6.4) on receipt from their suppliers (Sigma Chemical Co, Poole, or Supelchem, Sawbridgeworth in the case of PG) to ensure batch to batch continuity and to determine the fatty acid composition prior to their use in a liposome. Table 6.13 shows the fatty acid distribution in a number of phospholipids used.

There were marked differences in the fatty acid distributions in the phospholipid standards. The PE preparation had the highest USFAR and contained a large proportion of arachidonic acid. The lowest USFAR among the natural phospholipid preparations was 0.84, shown by the bovine brain PS. Lyso-PS, prepared from bovine brain PS, had a value of 0.28. The synthetic dipalmitoyl PC, obtained from Sigma, contained only palmitic acid.

TABLE 6.13 FATTY ACID DISTRIBUTION IN PHOSPHOLIPIDS

Fatty acid	Bovine brain PS	Egg PE	Bacterial PG	Soya bean PI	Bovine brain lyso-PS	Bovine brain PC
14:0	0.07	0.23	-	-	-	-
16:0	1.24	3.55	33.65	31.75	-	31.14
18:0	52.67	29.35	6.89	13.02	76.59	21.95
20:0	0.27	0.12	0.6	-	0.6	0.31
22:0	0.29	0.13	0.8	-	0.95	0.39
14:1	-	-	-	-	-	-
16:1	0.43	0.76	2.11	1.36	0.45	0.85
18:1	28.67	17.82	22.88	8.50	14.25	34.31
18:2	0.36	8.97	7.70	32.33	-	1.31
18:3ω 6,9,12	0.11	0.19	0.40	-	-	0.15
18:3ω 9,12,15	0.47	1.17	7.43	4.90	0.35	0.62
20:1	3.58	3.22	3.21	0.67	1.44	1.70
20:2	0.65	0.85	-	-	0.10	0.39
20:3	0.22	0.57	3.90	-	-	0.31
20:4	1.08	25.55	0.29	0.25	2.34	2.86
22:1	0.89	0.87	-	-	1.35	-
22:2	0.12	0.41	0.47	1.88	-	1.08
22:6	9.22	6.24	0.49	5.37	1.60	2.36
24:1	-	-	0.08	-	-	-
USFAR	0.84	2.00	1.41	1.23	0.28	0.86

6.6 PREPARATION OF LIPOSOMES

Pure phospholipids in chloroform:methanol (95:5 v/v) were mixed in the required proportions. Cholesterol in the same chloroform:methanol mixture was also added at this stage. The preparation was thoroughly mixed then taken to dryness under a stream of oxygen-free nitrogen. The dried lipid was resuspended in saline and thoroughly mixed once again. The suspension was sonicated (Dawe Ultrasonic Bath model 6447A) for 5 minutes at a temperature five degrees higher than the transition temperature of that component of the mixture with the highest T_c. This was done by placing a thermostatically controlled heating element in the ultrasonic bath (Grant Instruments, Cambridge). The process was carried out under a stream of oxygen-free nitrogen.

Following sonication, the preparations were cooled to $5^{o}C$ for 15 minutes prior to use in coagulation studies. A representative sample of liposomes was tested for purity and a further sample fixed for electron microscopy (see fig 6.8).

6.7 PURITY TESTING OF COMPLETE LIPOSOMES

A sample of liposome suspension was extracted by the method of Bligh and Dyer (1959) and the lipids subjected to two-dimensional TLC as described (system 5). The appearance of fatty acids or lysophosphatides was taken to indicate that some degradation had occurred.

6.8 ELECTRON MICROSCOPY

Liposome preparations, thromboplastin and partial thromboplastin were fixed in 0.22 mol dm^{-3} cacodylate buffered glutaraldehyde (pH 6.5) and centrifuged at 6500 x g for 1 hour. The pellet was washed with buffer and post-fixed in 40 mmol dm^{-3} osmium tetroxide. Following a further washing step the preparations were dehydrated in a graded series of alcohols. In order to facilitate this procedure the liposome and activated partial thromboplastin preparations had to be embedded in 1% agarose. This was not necessary for the tissue thromboplastin reagents.

The dehydrated blocks were then infiltrated with propylene oxide and embedded in Agar 100 resin. Sections were cut on either an LKB IV or a Reichart OMU-4 ultramicrotome, stained with uranyl acetate and lead citrate and examined in an AEI EM 801 transmission electron microscope.

Sections of 50 - 60 nm thickness were normally cut except in the case of General Diagnostics Automated APTT reagent which contained colloidal silica particles necessitating section thickness of approximately 100 nm. Fig 6.8 shows an electron micrograph of a liposome preparation made as described in Ch 6.6.

Fig 6.8 (opposite) An electron micrograph of a liposome preparation.Magnification X 5000, scale bar 500nm.

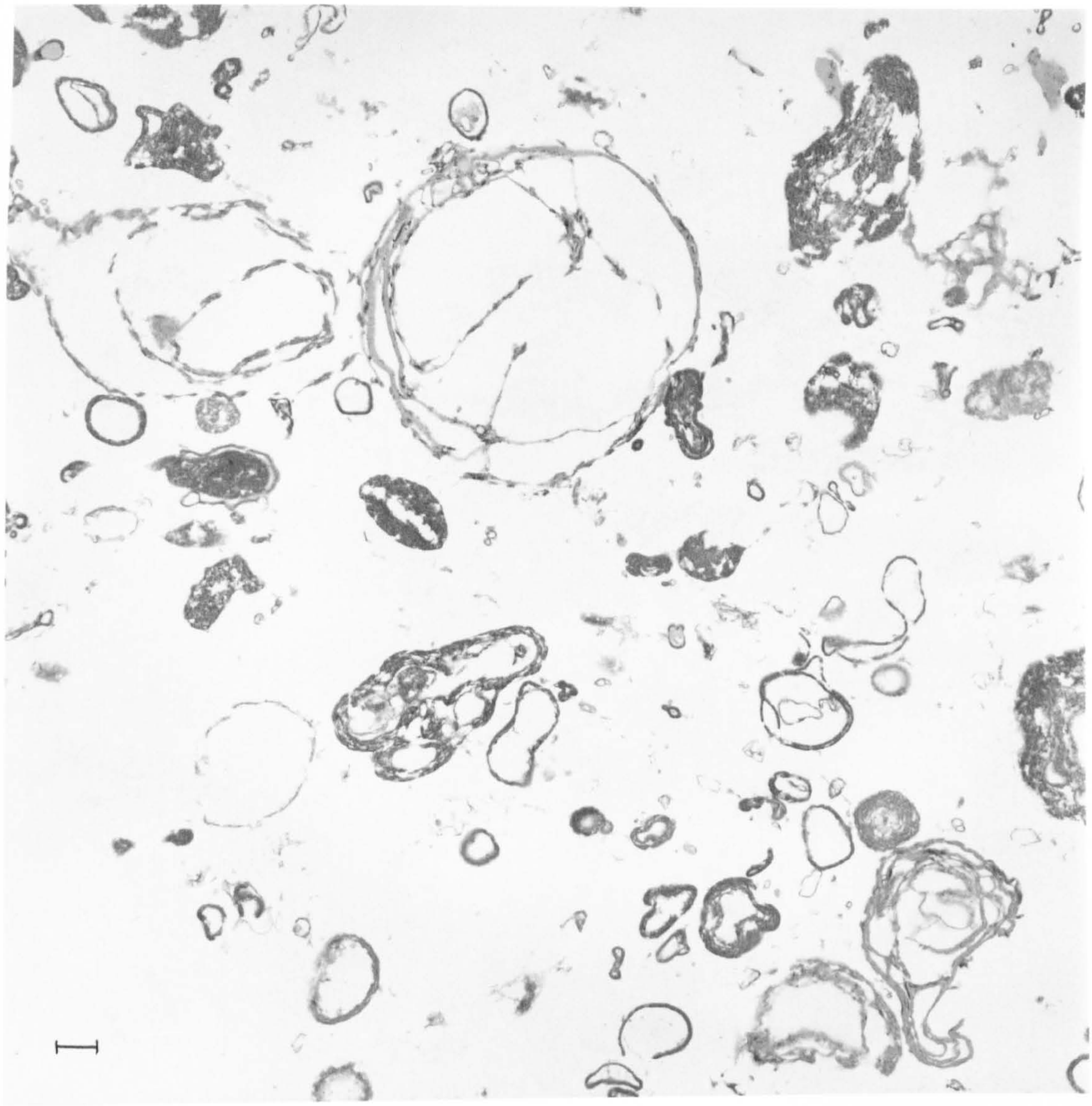


Fig 6.8

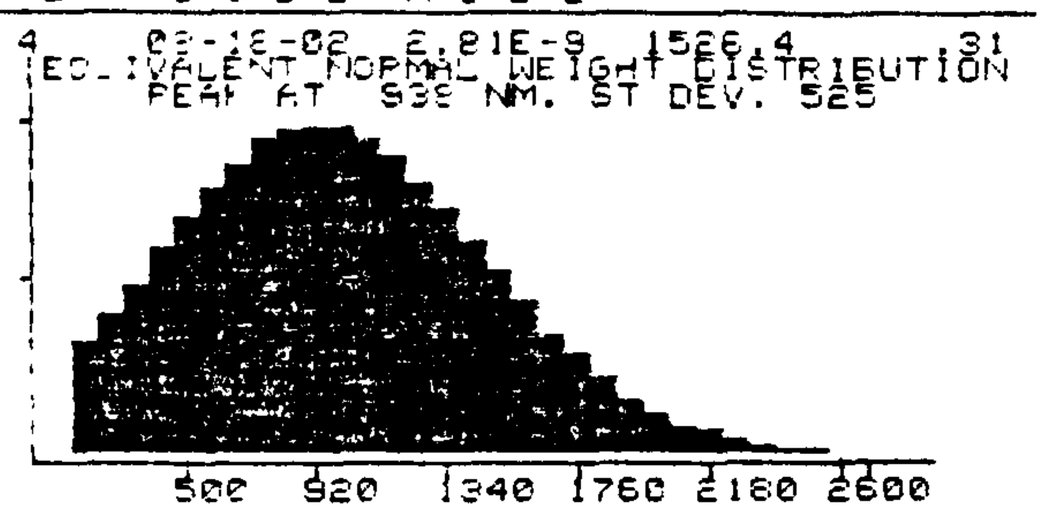
6.9 ESTIMATION OF SIZE DISTRIBUTION OF APTT REAGENT LIPOSOMES

Measurements of size distribution were made on Malvern Instruments Ltd. Autosizer or Zetasizer instruments which work on the same principle, as follows:

Particles suspended in liquid undergo random Brownian motion according to their size, smaller ones moving more rapidly than larger ones. The light scattered by the particles when they are illuminated by a low power laser beam fluctuates in intensity as a result of this motion. The technique of autocorrelation spectroscopy interprets these fluctuations in terms of particle diameters. The Zetasizer instrument reports diffusion coefficient, mean particle size and polydispersity. The equivalent normal weight distribution is presented graphically with peak particle size and standard deviation data. The Autosizer instrument gives a graphic presentation of the particle size distribution by weight together with a detailed table showing the numbers of particles falling in particular size ranges. As described in Ch 8.2, a Zetasizer 2 instrument was used for the study of two APTT reagents, General Diagnostics Automated APTT and Manchester APTT, with a 0.7 mm bore capillary cell (PC3). For the other reagents, Actin, Actin FS and Activated Thrombofax, an Autosizer 2 instrument was used, with a 4 mm bore PC4 capillary. A typical Zetasizer 2 output for Manchester APTT is shown in fig 6.9, while fig 6.10 shows a typical Autosizer 2 output for Actin FS. The major advantages of using this method for sizing are that the sample measured may be many millions in a matter of seconds and there is no possibility of samples being degraded by any preparative process.

M A L V E R N Z E T A S I Z E R 2 - S I Z E M O D E

TEMPERATURE (C) = 20.00
VISCOSITY (CP) = 1.0000
REFRACTIVE INDEX = 1.33
CELL TYPE = CAPILLARY
TIME OF DAY = 08-20-38



ENTER NEXT INSTRUCTION PRINT DEF:

M A L V E R N Z E T A S I Z E R 2 - S I Z E M O D E

TEMPERATURE (C) = 20.00
VISCOSITY (CP) = 1.0000
REFRACTIVE INDEX = 1.33
CELL TYPE = CAPILLARY
TIME OF DAY = 08-21-13

RUN NO	START TIME	DIFF COEFF	SIZE (NM)	POLY DISP.
	SET OF 3 EXPERIMENTS			
2	08-12-10	2.68E-9	1599.3	.43
3	08-15-45	3.11E-9	1380.8	.36
4	08-18-02	2.81E-9	1526.4	.31
MEAN		2.87E-9	1502.2	.373
STD. DEV.		2.18E-10	111.22	.060

ENTER NEXT INSTRUCTION PRINT DEF:

Fig 6.9 Size distribution of liposomes of Manchester APTT reagent.

M A L V E R N ' A U T O S I Z E R I I ' A N A L Y S I S R E P O R T

* MEDIUM RESOLUTION MODE *

SAMPLE TITLE :

FILE TITLE : NOT SET

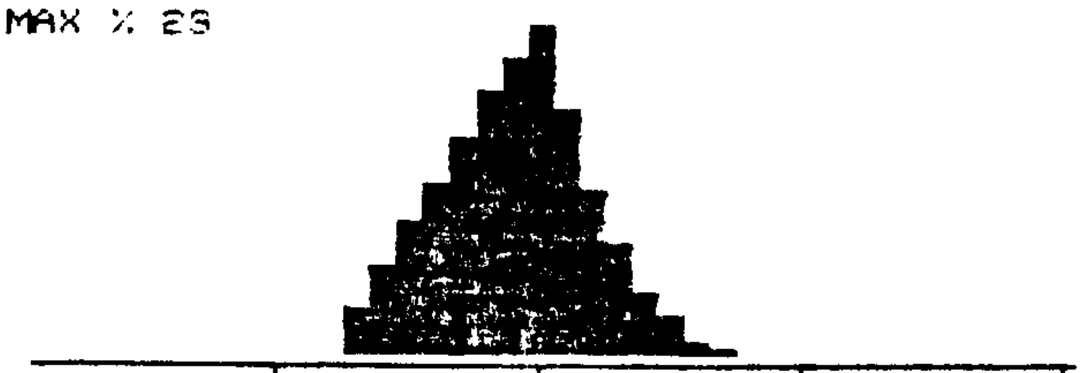
REFRACTIVE INDEX 1.33 VISCOSITY .9995 TEMPERATURE 20.1

PARTICLE SIZE DISTRIBUTION (NM.)

MEAN: 269.

MAX % 23

(BY WEIGHT)

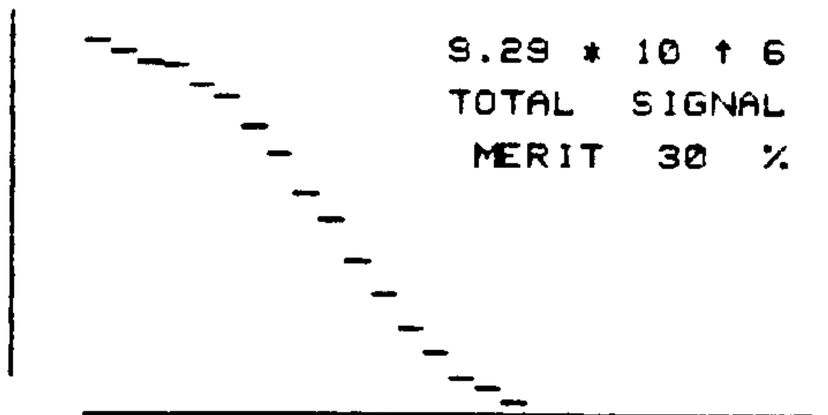


CORRELATION FUNCTION

9.29 * 10 ↑ 6

TOTAL SIGNAL

MERIT 30 %



TIME REMAINING : 0 SECS : OUT OF RANGE %: 5.04 : T 15 :P 20

SIZE RANGE			% WEIGHT, BELOW		% NUMBER, BELOW		INTENSITY
25.6	-	32.2	0 , 0		0 , 0		0
32.2	-	40.6	0 , 0		0 , 0		0
40.6	-	51.1	0 , 0		0 , 0		0
51.1	-	64.4	0 , 0		0 , 0		0
64.4	-	81.0	0 , 0		0 , 0		0
81.0	-	102.	0 , 0		0 , 0		0
102.	-	128.	0 , 0		0 , 0		0
128.	-	161.	8.19 , 0		9.42 , 0		27.4
161.	-	203.	15.7 , 8.19		18.0 , 9.42		52.5
203.	-	256.	24.1 , 23.8		27.7 , 27.4		80.7
256.	-	322.	29.8 , 48.0		34.3 , 55.1		100
322.	-	406.	14.9 , 77.8		8.61 , 89.5		99.9
406.	-	511.	5.77 , 92.8		1.66 , 98.1		76.9
511.	-	644.	1.40 , 98.5		.202 , 99.7		37.3
644.	-	810.	0 , 100		0 , 100		0
810.	-	1020	0 , 100		0 , 100		0
1020	-	1285	0 , 100		0 , 100		0
1285	-	1618	0 , 100		0 , 100		0
1618	-	2037	0 , 100		0 , 100		0
2037	-	2564	0 , 100		0 , 100		0

RUN NO.	START TIME	MEAN SIZE WEIGHT	NUMBER	COUNT RATE (*1000 /SEC)
2	00 : 04 : 07	269.	248.	130.

COMMENTS :

Fig 6.10 Size distribution of liposomes of Actin FS.

Discussion of methods available for estimation of size distribution

The method of negative staining with electron microscopy was found to be satisfactory for the analysis of small unilamellar vesicles giving values that were within 10% or 20% of values obtained by freeze fracture, hydrodynamic studies and nuclear magnetic resonance (Bergelson 1979, Huang 1969, Watts et al 1978). Olson et al (1979) discuss the difficulties encountered in applying this method to multilamellar vesicles. Since these consist of a heterogeneous distribution of sizes and shapes, for example large elliptical or cylindrical structures are observed, it was necessary to take the mean of the major and minor axes of such structures and assume that they had originally formed a sphere. The discrepancy between this method and freeze fracture in estimating vesicle size was the greatest for distributions that contained the largest diameter particles and the greatest heterogeneity. A technical difficulty in obtaining good negative stains of liposomes is the spreading of the vesicles on carbon-coated grids. Gregory and Pirie (1973) treated grids with bacitracin and found satisfactory spreading resulted. Freeze fracture has been applied to morphological studies of liposomes (Luna and McConnell 1977, Van Dijck et al 1976) but suffers from the serious problem that the fracture plane does not necessarily go through the middle of the liposome (Gruner et al 1985). This reduces the usefulness of the method for undertaking size measurements. Guoit et al 1980 employed mathematical methods to try to correct for this feature. The

problem of sampling size is of major concern in microscope techniques. Sufficient samples must be taken to exclude any possible bias. Gruner et al (1985) believed that 200 measurements was the minimum acceptable in freeze-fracture analyses.

Other methods to obtain size distribution include analytical ultracentrifugation (Huang 1969), nuclear magnetic resonance (Bergelson 1979) and quasi-elastic laser light scattering (Selser et al 1976).

Huang (1969), Huang and Charlton (1971) and Mason and Huang (1978) described hydrodynamic techniques in the analysis of small unilamellar vesicles. Gel chromatography on large pore agarose gels was described by this group in 1969, for separation of small unilamellar vesicles from multilamellar vesicles. Estimates of sedimentation coefficients can then be made. Huang and Charlton (1971) describe sedimentation analysis using mixtures of hydrogen and deuterium oxide.

Kirkland et al (1982) described a method which they called exponential-field sedimentation field flow fractionation (SFFF). In a specially designed channel, rotated in a centrifuge, dissolved or suspended solutes migrate according to their density, relative to that of the mobile phase which is flowing continuously through the channel. Smaller particles elute from the channel first, followed by components of increasing mass.

Gel-exclusion chromatography has been reported using Sephacryl S1000 (Reynolds et al 1982) and high performance liquid chromatography (Ollivon et al 1986). A drawback to both of these however, is the problem of calibration of the columns. The hplc technique was very quick, taking only 10 - 20 minutes to run.

The use of nuclear magnetic resonance to determine size depends upon the use of paramagnetic ions to shift or broaden the NMR signal from the choline trimethylammonium protons on the phosphate moiety of the phospholipid (Bergelson 1979). Use of the technique depends on the assumption that vesicles are unilamellar, spherical and of a defined bilayer thickness. Gerritsen et al (1979) demonstrated that the method could be used to show whether a population of large unilamellar vesicles was strictly unilamellar or whether it contained any multilamellar vesicles.

Finally, the use of light-scattering techniques has been described by several groups. Bangham et al (1974) used such a method and Chong and Colbow (1976) obtained size and heterogeneity estimates for polydispersed vesicle preparations. Aurora et al (1985) used an argon ion laser as the light source. The intensity of the light scattered at 90° was measured and diffusion coefficients calculated on a DEC PDP-8 minicomputer and a VAX 11/780 mainframe computer. Calibration was by the use of latex beads. Cornell et al (1986) describe a similar, though less complex, arrangement for sizing and the Malvern Instruments devices employed in this study use low power He-Ne lasers in conjunction with "desk-top" size computers.

6.10 THE MEASUREMENT OF ELECTROPHORETIC MOBILITY OF APTT REAGENT LIPOSOMES

The Zetasizer 2 instrument (Malvern Instruments Ltd, Spring Lane South, Malvern) was used for this task. It functions as follows:- at the crossing point of two beams derived by splitting the output of a He-Ne laser, a pattern of interference fringes is formed. Particles moving through this region scatter light whose intensity fluctuates with a frequency related to the particle velocity. This light is collected onto a photomultiplier system and processed to produce a short pulse for each photon detected. The correlator collects and averages this train of signals and the resulting "correlator function" is analysed to determine the frequency spectrum which is directly related to the velocity of the particles. Since the applied electric field is known, the electrophoretic mobility can be calculated. The complete measurement takes only seconds but is made over a sample of many millions of particles; an impossible feat with manual methods. It therefore represents a comprehensive and accurate assessment of the complete sample and the mobility spectrum of all its constituent particles. A printout showing the mobility data for Actin FS is shown in fig 6.11.

GRAPHIC RESULTS RUN 7 : MOBILITY SPECTRUM MEAN = 2.70 : % WIDTH 13.2

FREQUENCY : 100 252 405 557 710 862 HZ:
MOBILITY : 1.97 - .033 - 2.04 - 4.05 - 6.06 - 8.07 MB:

TABLE OF EXPERIMENTAL RESULTS

RUN NO	TIME	FREQUENCY (HZ)	MOBILITY MU/SEC/V/CM	WIDTH %	ZETA (MV)	LIMITS	COUNT RATE
SET OF 3 MEASUREMENTS							
5	14-51-40	454.	- 2.69	12.2	- 38.6	20; 29;	132.
6	14-54-05	454.	- 2.70	13.5	- 38.7	20; 30;	135.
7	14-56-30	455.	- 2.70	13.2	- 38.9	19; 30;	130.

MEAN MOBILITY OVER 3 RUNS :- 2.70 +/- 9.1E-3

PERCENTAGE MOBILITY TABLE

CHANNEL	MOBILITY	% IN BAND			
1	1.97	0	0	.559	2.05
2	1.43	25.10	9.86	1.00	9.22
3	.909	.0120	16.5	6.31	2.03
4	.376	0	0	0	0
5	-.157				
6	-.690				
7	-1.22				
8	-1.75				
9	-2.28				
10	-2.81				

MOBILITY INCREMENT PER CHANNEL - .200

RUN NO	TIME	FREQUENCY (HZ)	MOBILITY MU/SEC/V/CM	WIDTH %	ZETA (MV)	LIMITS	COUNT RATE

MEAN MOBILITY OVER 3 RUNS :- 2.70 +/- 9.1E-3

M A L V E R N Z E T A S I Z E R 2

ELECTROPHORESIS SYSTEM SOFTWARE VERSION EP1

SAMPLE TITLE :

SAMPLE DETAILS

CELL VOLTAGE (AUX)	ELECTRODE SPACING (MM)	VISCOSITY (CP)	DIELECTRIC CONSTANT
85	25	1	80

ZERO MOBILITY SET FREQUENCY -250 (HZ).

AUTOMATIC PRINTOUT

OFF

Fig 6.11 Mobility spectrum of Actin FS.

6.11 TESTING COAGULANT ACTIVITY OF LIPOSOMES

The activated partial thromboplastin time (APTT) was used as a measure of coagulant activity. The APTT of a fresh normal plasma was determined using a batch of the Manchester APTT reagent (batch 117). This normal time was assigned a value of 100%. Buffer or saline was substituted for the APTT reagent and the test repeated. This APTT result was assigned a value of 0%. The test liposome was then substituted for batch 117 and the resulting APTT converted to % activity of 117.

6.12 FREEZE-DRYING

Note on pressure measurement - manufacturers of vacuum equipment and related apparatus calibrate gauges in millibars not the SI unit which is Newtons per square metre (1 mb = $10^2 Nm^{-2}$).

Apparatus

Fig 6.12 shows a freeze-drying plant at the UK Reference Laboratory for Anticoagulant Reagents and Control. The plant was a Polyvac PVFD 6 x 4, built by Jones Bros, Bury, to specifications decided in collaboration with the author. It comprised four identical chambers which could be independently isolated, thereby allowing the drying of any volume up to approximately 18 dm^3. The maximum drying capacity of a freeze-drying plant depends upon the capacity of the refrigerated condensing surface used to entrap subliming vapour. Ours had a capacity of 18 dm^3 and was operated using either a mechanical refrigeration system, employing a 2.25 kJs^{-1} compressor, capable of cooling the condenser to -65°C, or a liquid nitrogen cooled system capable of cooling to lower than -190°C.

Each chamber contained six shelves (fig 6.13) which were adjustable to accept vials up to 47 mm in height. When required, two shelves could be removed from the assembly to accommodate vampoules of 64 mm height. For bulk drying, all six shelves could be removed.

Fig 6.12 (opposite) Freeze drying apparatus.

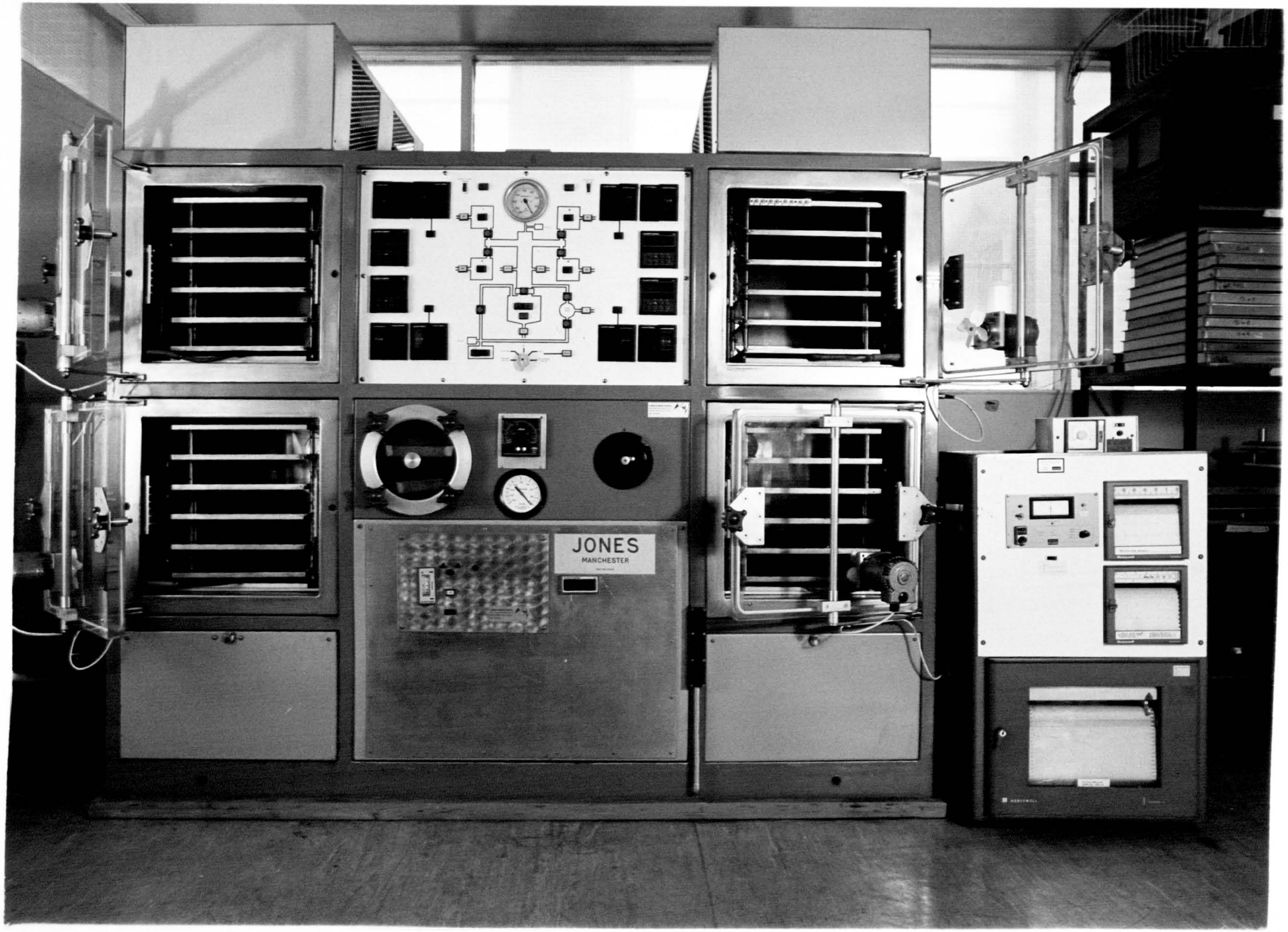


FIG 6.12

Each shelf had an integral heating element and a thermocouple for measurement of shelf temperature. In total, 3.6 kJ/s of heat were available, i.e., if all chambers and shelves were in use 150 J/s were available into one shelf. A reliable guide is 1 J/s dries 1 ml in 1 hour (Rowe and Snowman 1976). On this basis the plant would theoretically be able to sublime 18 litres of ice in 5 hours.

Chamber 3 was modified slightly, to accommodate a connecting strip, which can be seen to the top left of fig 6.13. Into this strip were plugged resistance bulb thermometers, randomly placed in the load, and the resistivity probe, for control of heating. This is described in some detail below.

One resistance bulb was connected to the chart recorder on the top right of the trolley shown in figure 6.12. Six other resistance bulbs were connected into the temperature indicator seen on top of the trolley in fig 6.12. A further chart recorder was used to record the following parameters -

1. shelf temperature (from a resistance bulb sensor placed in a hole drilled in shelf number 3 in chamber number 3).
2. condenser temperature (from a resistance bulb sensor strapped to the condenser coil).
3. load temperature
4. load temperature

These were from resistance bulb sensors placed randomly in the load.

5. pressure at head 1 (P1 in fig 6.15)
6. pressure at head 2 (P2 in fig 6.15)

Fig 6.13 (opposite) Detail of freeze drying chamber.

FIG 6.13

These two readings were from a Pirani vacuum gauge which provided output in the 0-10 mV range from each of two sensing heads. One of these was placed in close proximity to the vacuum pump, the other in a position at the centre of the pipework connecting the four freeze-dryer chambers. The other pressure gauge (P3 in fig 6.15) was of the Bourdon type and gave an indication of the pressure in the system, useful when pumping a "rough" vacuum.

A further pressue gauge of the Bourdon type (obtained from Bailey and Mackey, Birmingham) was subsequently fitted to the machine. This was to measure pressures in the range from atmospheric pressure to 800 mbar. This gauge was therefore accurately calibrated over this range, while being robust enough mechanically to withstand being subjected to a high vacuum. The purpose of the gauge was to indicate pressure when breaking vacuum with nitrogen or argon after a freeze-drying run, in order to introduce a partial pressure of one or other of these gases into the freeze-drying vials, prior to sealing.

Each of the four chambers had separate switching and thermostatic control of the heating supply, and one master control (black knob at centre right of fig 6.12) determined the current available for heating.

Resistivity control of freeze-drying temperature

The technique of resistivity measurement of lowest eutectic temperature is outlined below.

Consideration of a hypothetical freeze-drying operation will illustrate how this measurement may be of practical use. Suppose a solution of sodium chloride is to be freeze-dried. The lowest eutectic temperature, determined by resistivity measurement, was $-22^{o}C$. This means that during the primary drying stage the temperature must not be allowed to rise above $-22^{o}C$, the temperature of "incipient melting". From the warming curve (broken line fig 6.22) it may be seen that at $-22^{o}C$, the temperature of incipient melting, the resistance of the frozen NaCl solution falls very quickly indeed. Thus, if melting is to be avoided, the temperature must be maintained below $-22^{o}C$, or alternatively, the resistance must be maintained above 10,000 ohms. The resistivity controller, which is continually monitoring resistance, contains a relay which is able to switch the heating circuit of the freeze drying machine on and off, thereby controlling the heat input keeping it at all times below the level which would cause melting. The resistivity controller may be seen at the top left of the trolley in fig 6.12.

Some considerations on condenser design

As discussed in Chapter 5, a number of authors have examined condenser design and efficiency. Rowe (1964b) believed that the surface area of the condenser should equal that of the shelves, although as little as half this surface may be adequate. In our freeze-drying plant, the shelf area is 3.88 m^2. The condenser is a 22 m length of 16 mm outside diameter copper pipe, formed into a

helical shape with the tubing spaced so as to allow an ice layer thickness of 20 mm. The surface area of this condenser is 1.11 m^2 or 28.6% of shelf area.

The mechanically cooled condenser was replaced by a liquid nitrogen (LN_2) cooled model for a number of experiments described below (fig 6.14). Careful design of this apparatus was essential, to avoid excessive pressure build up within it.

The liquid nitrogen cooled condenser was made from 316 grade stainless steel. Two plates were formed into cylinders, one of 321 mm outside diameter, the other of 305 mm. These were welded together, top and bottom, and joined by 3 rows of ten "ties" in the form of lengths of 13 mm tubing welded in place. The nitrogen coolant was admitted, via a solenoid valve, through a 13 mm pipe which was connected to a 250 litre liquid nitrogen dewar. Exhaust nitrogen vapour emerged through a 32 mm pipe to the outside of the building. A thermocouple of copper/constantan placed in the exhaust of the condenser maintained the LN_2 level within it by switching the solenoid valve when the temperature exceeded the set value. This method of construction resulted in a very strong vessel able to withstand the rigours of very low temperature use. Its surface area was 0.74 m^2 or 19.07% of shelf area.

The temperature of the liquid nitrogen cooled condenser could be set at any level down to -190°C. When defrosting, care had to be taken to raise the temperature slowly, so that any residual liquid nitrogen inside did not vapourize too violently (1 cm^3 of

Fig 6.14 (opposite) Liquid nitrogen cooled condenser.

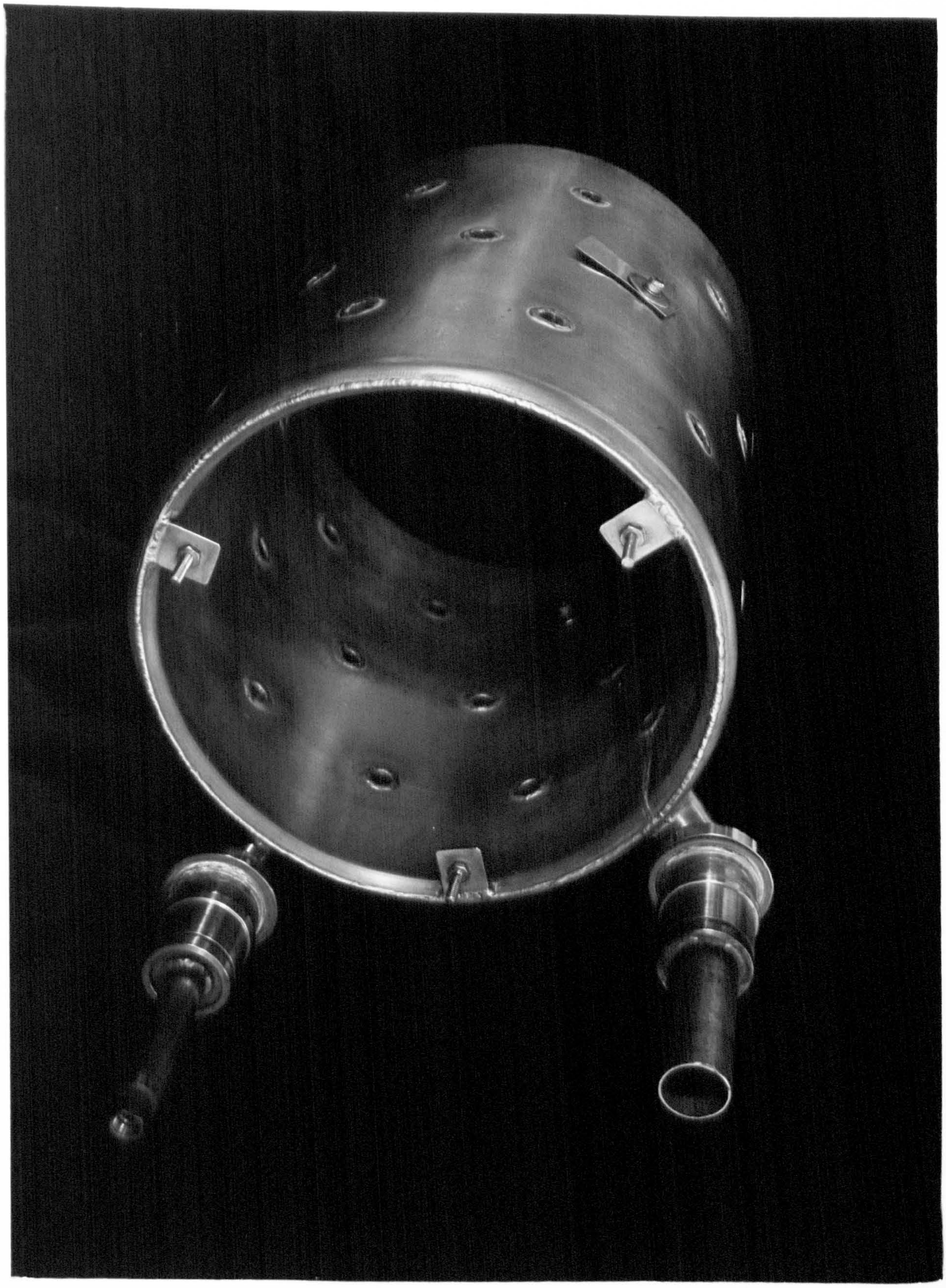


FIG 6.14

liquid nitrogen will expand to 680 cm^3 of vapour). A further consideration was that extreme changes in temperature could weaken the welding holding the condenser together. Accordingly, the condenser was first cleared of liquid nitrogen by "blowing" through with nitrogen gas.

The condenser temperature was then allowed to rise to about $-100^{o}C$ before flooding with water at $45^{o}C$ to clear the ice. (In the mechanically cooled condenser, the lowest temperature attained was $-68^{o}C$, so that defrosting was easily performed by flooding with warm water, without any likelihood of damage.)

Precooling of chambers

Each chamber of the freeze-drying plant was able to be independently cooled, before loading. A 250 Js^{-1} freezer unit, charged with R502, was provided for each chamber, enabling shelf temperature to be controlled, by means of thermostats, down to $-25^{o}C$. Fig 6.13 shows the door mounted fan unit, necessary for circulation of the cold air in the chamber, and the two cold "radiators" may be seen on either side of the shelf assembly.

Operation of the freeze-drying machine

(Labels refer to fig 6.15, a schematic diagram of the apparatus). 24 hours before loading, the plant was switched on by starting the vacuum pump and condenser. Valve V_5 was opened and

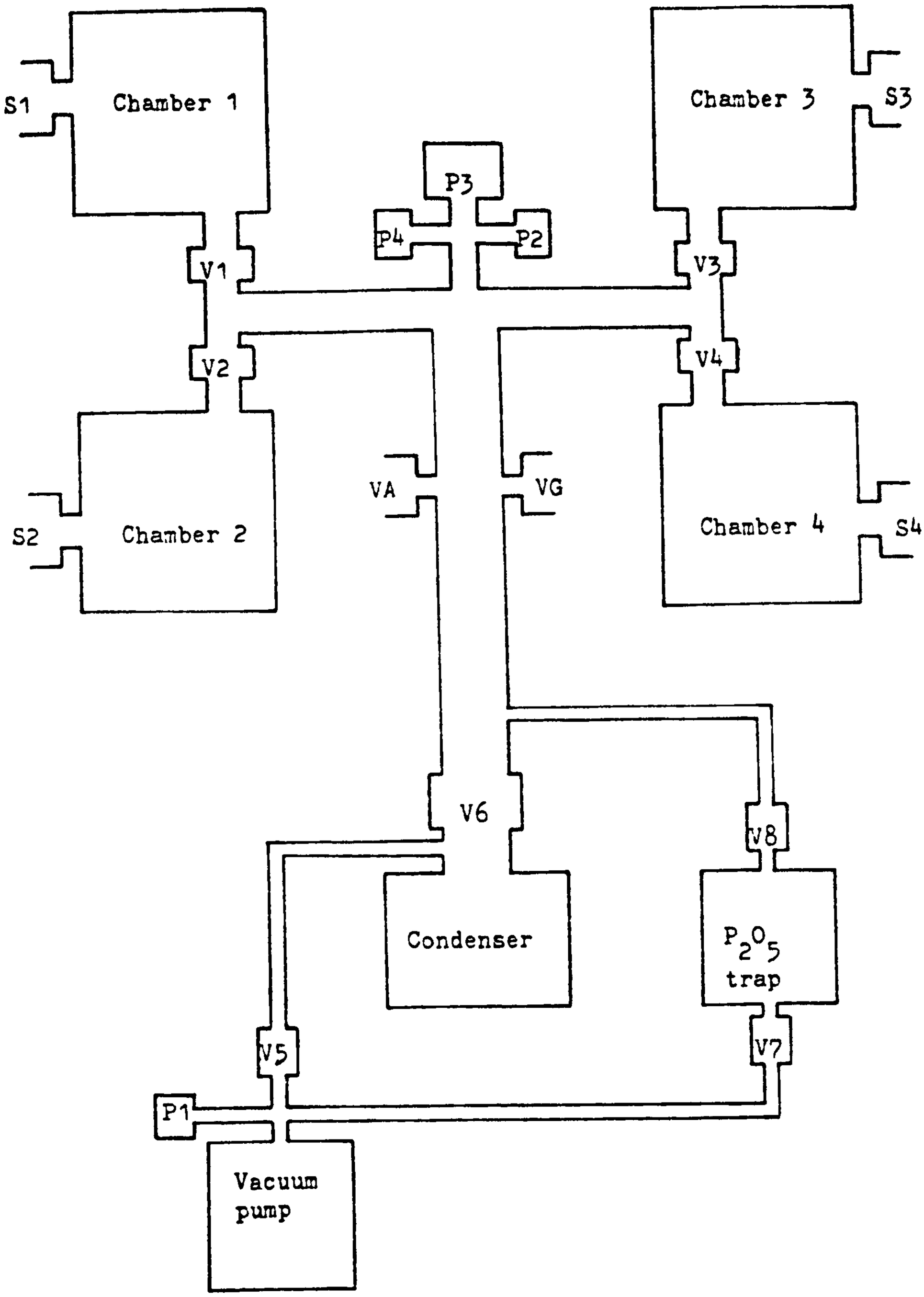


Fig 6.15 Schematic diagram of freeze drying plant.

the plant allowed to run in this stand-by condition until loading. Any necessary pre-cooling of chambers was also undertaken at this stage. Valves V_1, V_2, V_3 and V_4, were opened if the run required the use of all four chambers.

The material to be freeze-dried was then loaded, in the frozen state, from a "holding freezer" running at -80°C (Kelvinator model UC517, Laboratory Impex Ltd, Teddington). Any connections, of resistance bulb thermometers and resistivity probes, which were necessary, were made at the time of loading. The chamber doors were then clamped and the plant switched to the primary drying mode. In this mode, valves V_1 to V_6 were open. Subliming vapours were drawn toward the vacuum pump and were condensed on the cold surface of the condenser unit. Vacuum gauges were situated at P_1, P_2, P_3 (Pirani type) and P_4. P_3 and P_4 (Bourdon type) were used at a later stage, while P_1 and P_2 were referred to throughout the primary and secondary stages of drying. P_1 indicated the pressure at the pump, P_2 indicated the pressure in the pipework in proximity to the chambers. While sublimation was proceeding at a high rate, P_2 showed a higher pressure. This difference in pressure was an indication of the progress of the freeze-drying process. When primary drying was complete, i.e., when sublimation was complete, no further vapour was evolved so P_1 was equal to P_2.

A second important observation was the change in the temperature of the material being dried. After sublimation, the

material should be at ambient temperature, indicating that no ice was present in the material.

At this stage the plant was switched to the secondary drying mode. V_5 and V_6 were closed and V_7 opened. After a 30 second delay, to evacuate the P_2O_5 trap, V_8 was opened. In this mode, desorbing vapour was held by the desiccant. Desorption was aided by the application of heat to the material. At this stage the condenser was defrosted. Indications that secondary drying was complete were, once again, the material temperature, which should have reached the set value, and the pressure in the system, i.e., P_1 was equal to P_2. If necessary, during secondary drying, the P_2O_5 trap may be isolated and recharged.

When drying was complete the vials were sealed in vacuo. To do this, valves S1 to S4 were opened, allowing pure, dry nitrogen to be drawn in to an inflatable bladder, situated in each chamber at the bottom of the shelf assembly. The inflating bladder compressed the shelves upwards pushing the stoppers firmly home.

Valve VA was then opened, allowing the chambers to fill with air to atmospheric pressure. Following this operation, the plant was emptied, cleaned and made ready for the next drying sequence. Alternatively, vials were sealed in a partial pressure of either nitrogen or argon. The selected gas was introduced by opening valve VG, which allowed the vacuum to be broken and a partial pressure, indicated on gauge P_4, obtained. The vials were then sealed, as described above.

The freeze-dried material was then subjected to a number of tests, i.e., a random sample was removed for testing of inter-vial variation and measurement of residual moisture.

The random sampling of vials for inter vial variation was as described by Stevenson (1978). Measurement of residual moisture and the difficulties of sealing of vials and testing for leaks are discussed below.

This description applies to the "standard" lyophilisation method, employing a mechanically cooled condenser during sublimation and P_2O_5 as desiccant during desorption (see Chapter 9.1).

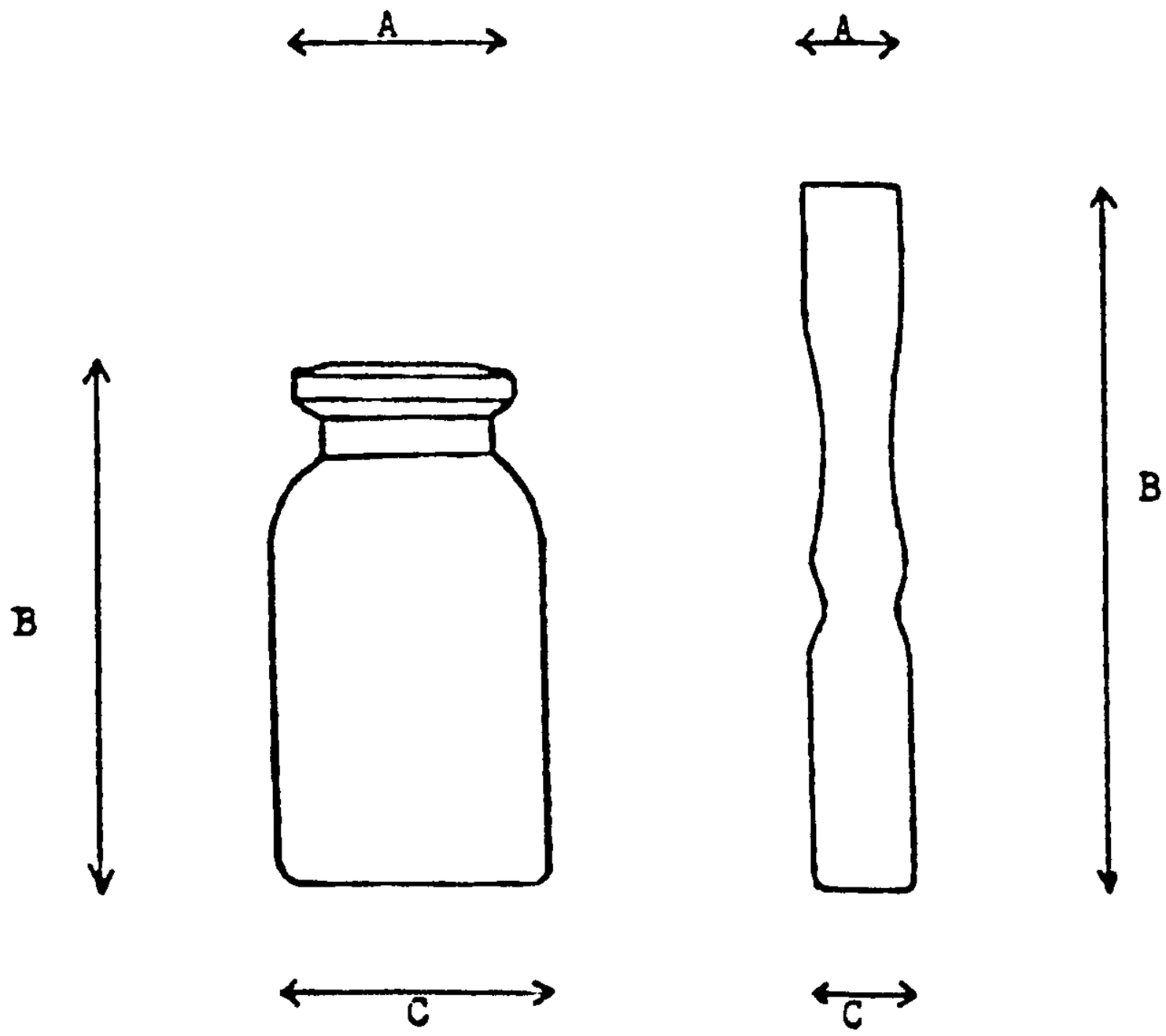
An alternative to the use of P_2O_5 during desorption was a liquid nitrogen cooled apparatus. This took the form of a finned element, cooled by LN_2. LN_2 was pumped into a reservoir within the element, under the control of a thermostatically controlled valve. The fins, of nickel-plated brass, were designed to present the maximum possible cold surface within the trap. Excess LN_2 vapour was piped to the outside of the building. Fig 6.16 shows the design of the finned element. The temperature controller was obtained from Eurotherm Ltd, Warrington.

Vials, stoppers and overseals

Glass freeze-drying vials of 2 and 10 cm^3 nominal capacity were obtained from FBG Trident Ltd, Bristol (see fig 6.17). A polypropylene vial of 4 cm^3 nominal capacity was obtained from

Fig 6.16 (opposite) Liquid nitrogen cooled trap for desorption stage of freeze drying.

FIG 6.16



vial type	A	B mm	C	material
1	13	37	14	glass
2	9	64	9	glass
3	13	38	17	polypropylene
4	20	34	24	glass

Fig 6.17 enclosures used in freeze drying

Dougherty Bros, Buena, New Jersey. Finally, a 1 cm^3 nominal capacity "vampoule" was obtained from Verretubex, Nogent-le-roi, France. Butyl rubber stoppers were obtained from West Pharmarubber, St Austell in sizes to fit the glassware described, i.e., 9 mm for the vampoule, 13 mm for the 2 cm^3 glass and 4 cm^3 polypropylene containers and 20 mm for the 10 cm^3 glass vials. These were as shown in figure 6.18.

Rubber stoppered vials were provided with aluminium tear off overseals (West Pharmarubber, St Austell). These were crimped in place using a crimping vice powered by compressed air (Jones Bros, Bury).

Sealing of vampoules

A device for sealing of vampoules was manufactured by Jones Bros, Bury (see fig 6.19). Vampoules, containing thromboplastin, were stoppered in vacuo following freeze-drying, then placed one at a time into a rotating chuck, and held by a vacuum. The stoppered end of the neck of the vampoule was gripped firmly by a spring-loaded device which maintained a constant tension on the constriction as a flame was moved close enough to melt the glass. The flame was natural gas, supplemented with compressed air and adjusted to a very fine point. As the glass melted, the stoppered end was drawn upward and a seal was made as the tip of the vampoule rotated in the flame.

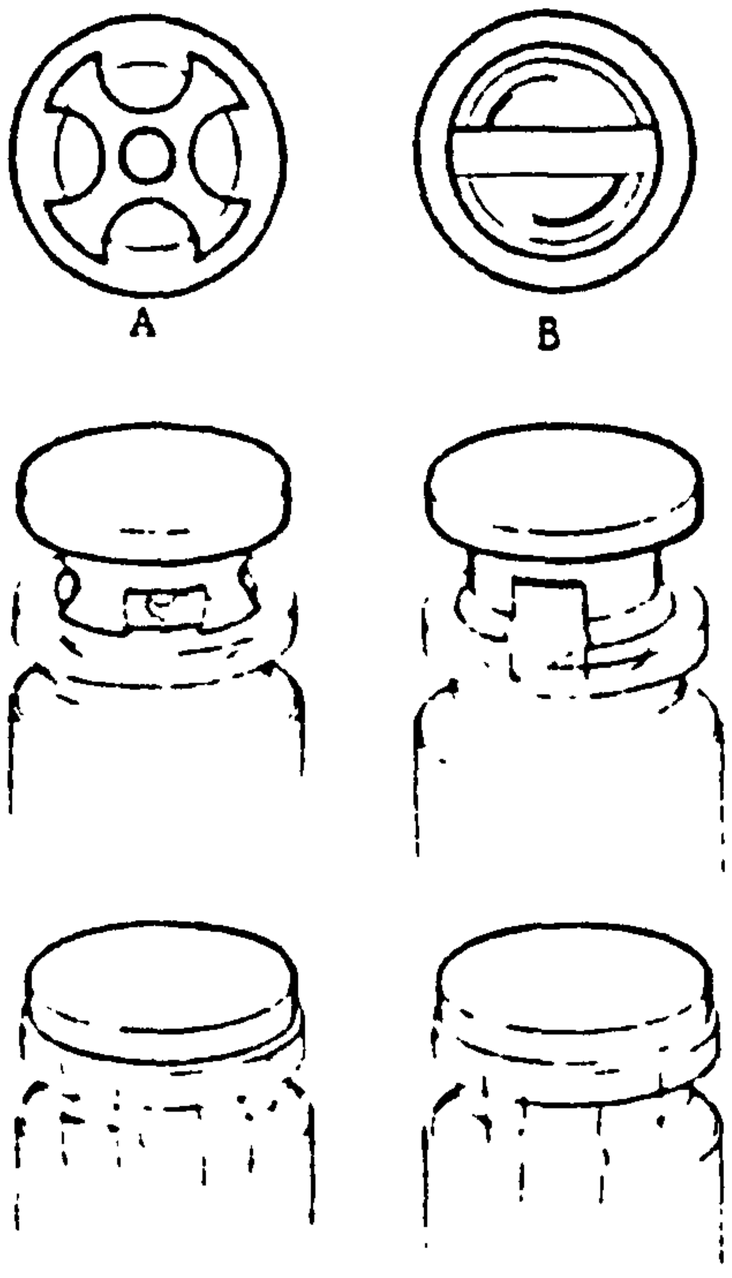


Fig 6.18 Rubber stoppers for freeze-drying vials showing stopper design and partially and fully stoppered positions.

Fig 6.19 (opposite) Device for flame sealing of vampoules.

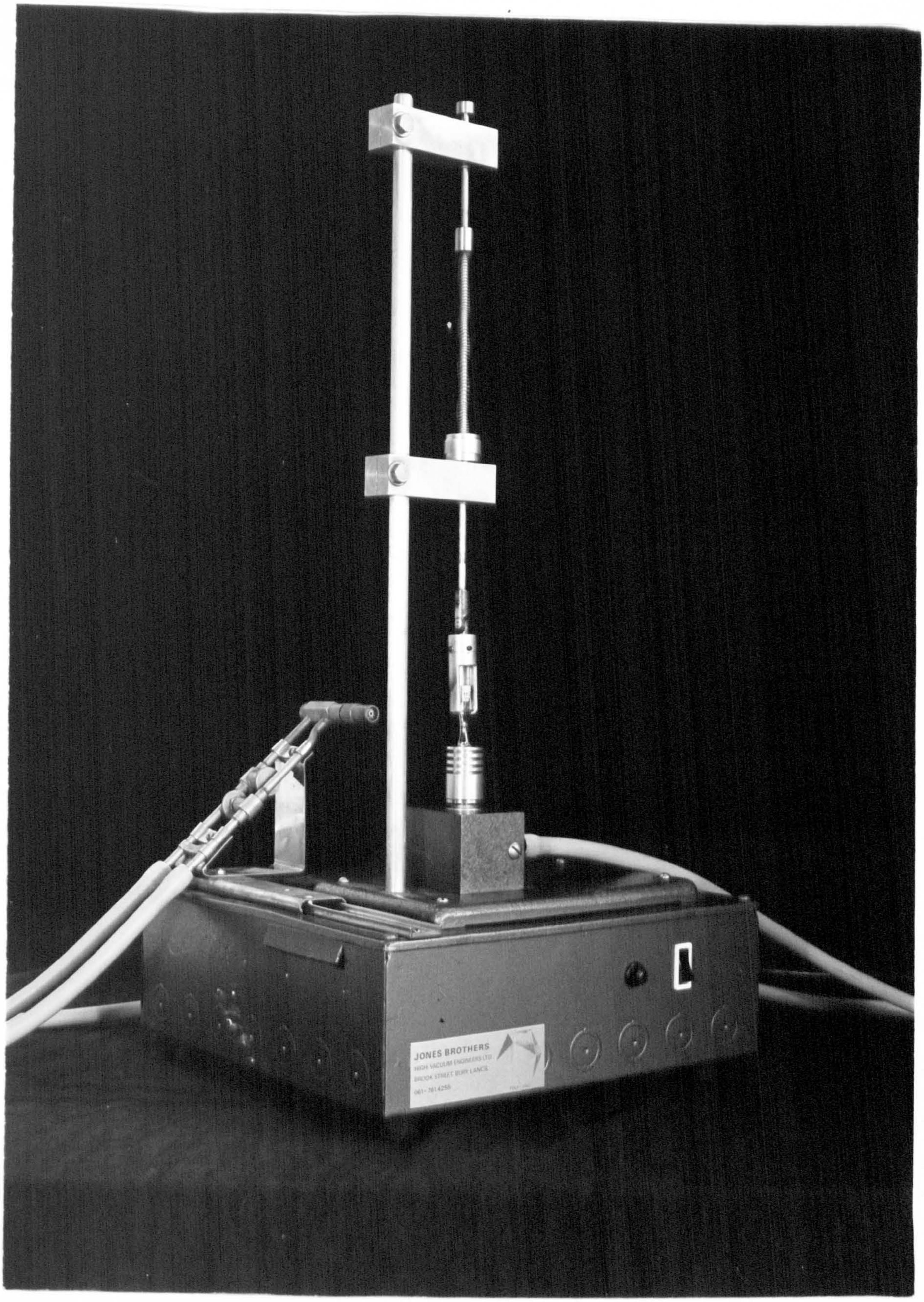

JONES BROTHERS
HIGH VACUUM ENGINEERS LTD
BROOK STREET BURY LANCS
061-761 4255

FIG 6.19

Several very important features of the process deserve further consideration. It was essential that the freeze-dried material in the vampoule must not be exposed to heat. This was ensured by making the chuck from a substantial piece of stainless steel with fins to improve heat transfer. The vampoule was held deep in the chuck so that the freeze-dried material was as far away from the flame as possible.

Following sealing, the vampoules were placed in an aluminium rack and allowed to stand at room temperature for twenty minutes. This allowed time for the glass to cool and avoided breakages which occurred if hot glass was placed at -20°C. Vacuum testing was then performed, as described below, before placing the sealed vampoules at -20°C.

Vampoules which had not retained their vacuum following stoppering were easily identifiable, since, on warming, enough pressure developed inside them to blow off their rubber stoppers.

Leak testing

The Edwards High Vacuum Model T2 high frequency spark tester was used for some experiments and was superseded by the Model ST4M when this improved model became available. The tester contained a Tesla coil, coupled to condensers which fed the output to an electrode. The output frequency was 3.8 MHz. In use, the electrode was passed over the surface of the vial under test which was

evacuated to a pressure of approximately 10^{-2} mbar. A discharge indicated that the vial was evacuated.

The spark tester worked efficiently if the pressure within a vial was less than approximately 100 mbar. No discharge was visible, however, if there had been leakage into a vial sufficient to raise pressure above this level. To determine whether there was still negative pressure in such vials the method of Barbaree and Smith (1981) was used. In this method, a 2 cm^3 syringe (Becton Dickinson and Co, Cowley, Oxford) was attached to a no. 23G1 needle and the plunger withdrawn to the 2 cm^3 mark. The needle was then inserted into the vial. A negative pressure in the vial caused the plunger to close. The method was used in this purely qualitative way, no attempt being made to use it to quantify the pressure in the vial.

Moisture determination

The method of choice was the "heat and weigh" method based on the technique described by Beckett (1954). An improved apparatus was built for this task by Jones Bros, Bury, according to specifications drawn up by the author, since the earlier method for residual moisture measurement described by Stevenson (1978) employed valuable freeze-dryer space for long periods. The new apparatus is shown in fig 6.20. Briefly, it comprises a glove box with a vacuum oven attached. Inside the glove box was a Mettler H80 balance. Air was drawn through a filter and dried in a column of molecular seive

Fig 6.20 (opposite) Apparatus for determination of residual moisture.

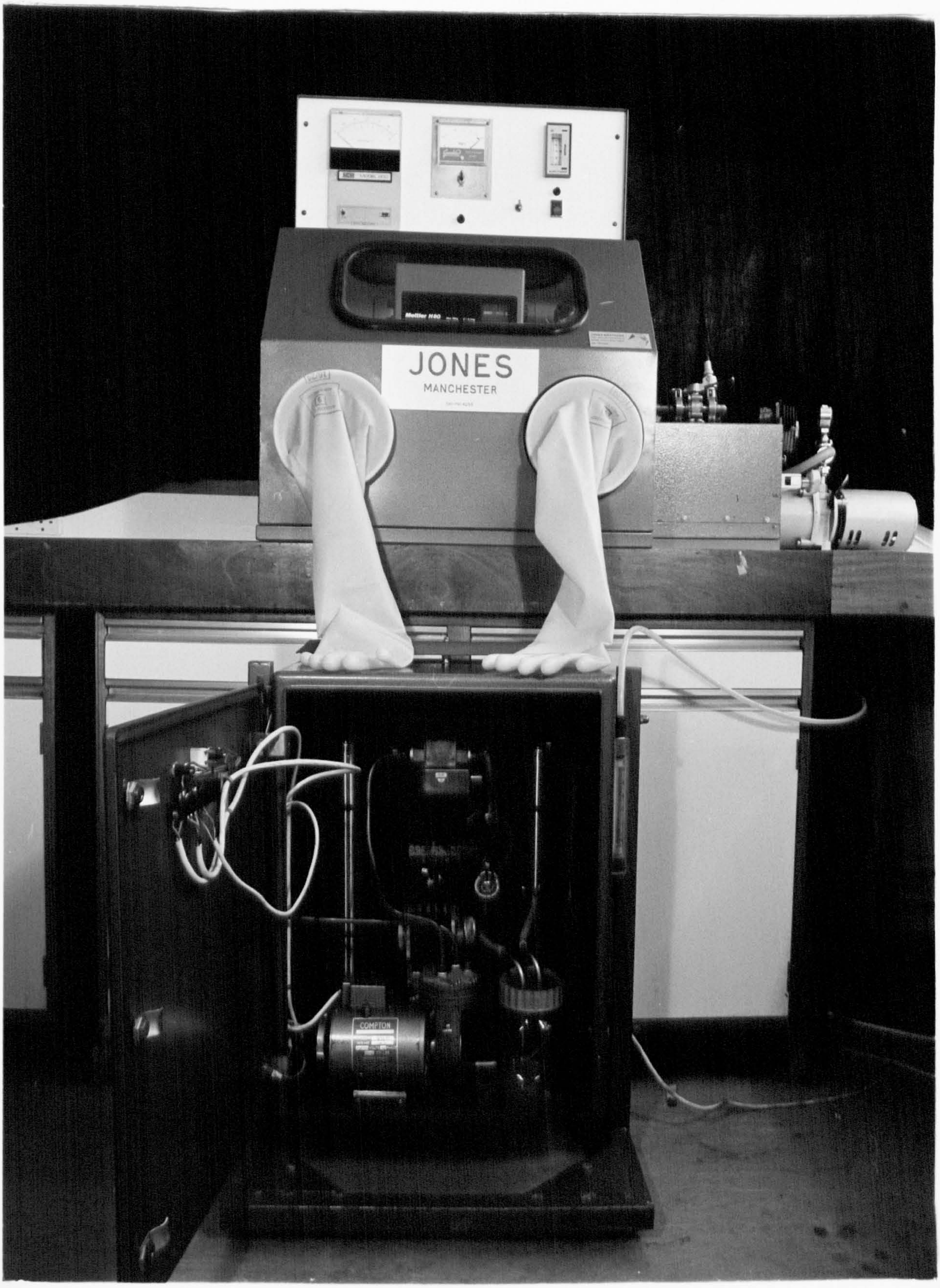


FIG 6.20

(type 4A Union Carbide) before being pumped into the glove box. There were two columns in the apparatus, one of which was recharged by heating while the other was in use. Switching from one column to the other was under the control of an interval timer device. Moisture extracted from the air was collected in the glass jar, to be seen at the bottom of the lower box. A one-way valve was situated at the back of the glove box so that excess air pressure was periodically relieved. A hygrometer monitored the relative humidity inside the glove box, which achieved a minimum dewpoint of approximately -58°C. This was equivalent to a relative humidity of 0.057% (at 21°C) or, more important, to a water vapour pressure of 0.013 mbar, which was approximately equal to the pressure that obtained in the freeze-drying plant at the end of a typical drying sequence.

The apparatus was switched on and allowed to equilibriate overnight. The vacuum oven temperature was set at 45°C. Vials of freeze-dried material, stoppered in vacuo, were placed in the glove box and allowed to equilibriate for one hour to dry off the outside of the vial. The stoppers were then withdrawn to the "partial stoppered" position as shown in fig 6.18, and the vials and their contents weighed. This weight was the "freeze-dried weight". The vials were then placed in the vacuum oven which was evacuated by connecting to a rotary pump (NGN model PD-2). After 24 hours, vials and contents were weighed and the weight recorded. The vacuum oven process was repeated until no further weight loss was noted. This weight was taken to be the "true dry weight". The difference between the "freeze-dried weight" and the "true dry weight" was

taken to be due to residual moisture. Vials and stoppers were then washed, oven dried and weighed to enable the weight of freeze-dried material to be calculated, and the residual moisture expressed as weight change/orginal freeze-dried weight x 100%.

Pemberton (1976) discussed a number of factors in the vacuum-oven technique which influence the estimation of moisture in freeze-dried materials. He was unhappy with reported methods in a survey of manufacturers of veterinary vaccines and cautioned that sufficient numbers of vials must be tested in order to arrive at a good estimate of the average moisture content of a batch. He believed that it is essential that manufacturers be aware of any "shelf-to-shelf" or "front-to-back" or "edge-to-middle" variations within their freeze-drying plant (see Chapter 9).

Another important area was the handling of vials throughout testing. Glass surfaces are avid desiccants (Pemberton 1976), so care was taken to dry off the outside of the vials before any weighing. Gloves were worn as a further precaution to prevent contamination of the glass surface.

The apparatus described here was able to accommodate large numbers of vials and since the entire process was confined to the glove box or the vacuum oven there was no danger of contamination by atmospheric moisture.

To calculate the coefficient of variation (CV) of the method, 20 lots of 5 vials were removed from a batch of lyophilised

thromboplastin and the residual moisture calculated. The mean value for the 20 observations was 2.10% with a standard deviation of 0.064. The coefficient of variation was 3.04%.

Temperature measurement

Platinum resistance temperature sensors were used. These were 1.59 mm diameter x 8 mm in length, to British Standard BS1904. (This standard is more exacting than the relevant German and American standards, DIN 43760, 1968 and RC21-4-1966) respectively). Platinum resistance temperature sensors were preferred for measurement of material temperature during drying, for a number of reasons. Firstly, no compensation was needed for junctions - theoretically any number of junctions in the sensor cable would have been tolerated - use of thermocouples would have been difficult for this reason.

A second advantage of resistance bulbs for this function was the fact that they were connected to the temperature indicating device by very fine wires, allowing them to be placed at any position in the freeze-drying load. Thermocouples are less flexible in use and are better suited to fixed positions such as monitoring of shelf temperature (see fig 6.13).

Resistance bulb sensors were periodically checked, using boiling water as the high temperature standard and solid CO_2 as the low temperature standard. Solid CO_2 was prepared in a Frigimat device obtained from Radley Ltd, Sawbridgeworth. This was

connected to a siphon cylinder of CO_2 and produced a dense pellet of solid CO_2 of some 450 g.

Resistivity measurement of lowest eutectic temperature

The conduction of electricity through a solution is dependent on the mobility of ions in the solution. If there is liquid present, the electric current can pass unhindered, but when the solution is completely solidified, i.e., at and below its lowest eutectic temperature, the ions are immobilized and the resistance of the material will be very high. Due to the differing degrees of movement and, hence, energy levels of molecules and ions in solids and liquids, there is an easily measurable difference in the electrical conductivity and hence resistance between solids and liquids. More precisely, the measurement depends on the resistivity of the test material, which is constant for a material at a particular temperature, but is not dependent on the physical size of the conducting medium, as is resistance. However, as during one measurement the same diameter probes with the same spacing between them are used, the resistivity is directly proportional to the resistance.

$$R = \rho \frac{A}{L}$$

where R = resistance

ρ = resistivity

A = presented probe area

L = distance between probes

As A and L are constant $R \propto \rho$

The equipment (Edwards High Vacuum Ltd) comprises a Wheatstone bridge circuit with the probes in one leg of the bridge and a variable resistor in the other as shown in fig 6.21.

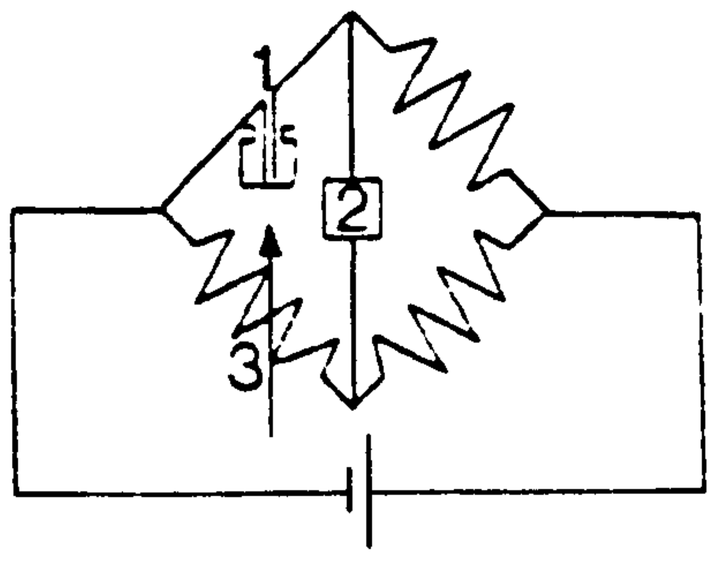


Fig 6.21 circuit for resistivity measurement.

1 = probe, 2 = balancing meter, 3 = variable resistance.

The probes were placed in a vial of test material and the vial cooled in a cooling mixture of industrial methanol and solid CO_2.

At various stages of cooling the temperature was recorded by means of a resistance bulb thermometer (Jones Bros, Bury) placed between the probes, and the variable resistance adjusted to obtain a balance on the galvonometer. This indicated that the adjustable resistance had the same value as the resistance of the test material. A complete set of readings was made to below the eutectic

temperature of the test material and again as the material was subsequently warmed.

The equipment was calibrated by measuring the eutectic temperatures of a number of electrolytes whose eutectic temperatures were known from tables (Meryman 1966).

TABLE 6.14 EUTECTIC TEMPERATURES (^{o}C) OF FOUR COMMON ELECTROLYTES

	values from graphs
KCl	-12
NaCl	-22
$MgCl_2$	-33
$CaCl_2$	-55

The curve for warming (broken line in graph) is the curve used for control of freeze-drying temperature. See fig 6.22.

Modification to the Comp-u-pet apparatus to enable dispensing into vampoules.

The glass vampules shown in figure 6.17 were designed to allow freeze-drying and stoppering in vacuo to be carried out in exactly the same way as when standard freeze-drying vials were used.

Following stoppering, however, they may be flame-sealed at the specially constricted neck. A major problem was the difficulty caused by this constriction when dispensing thromboplastin into the ampoule.

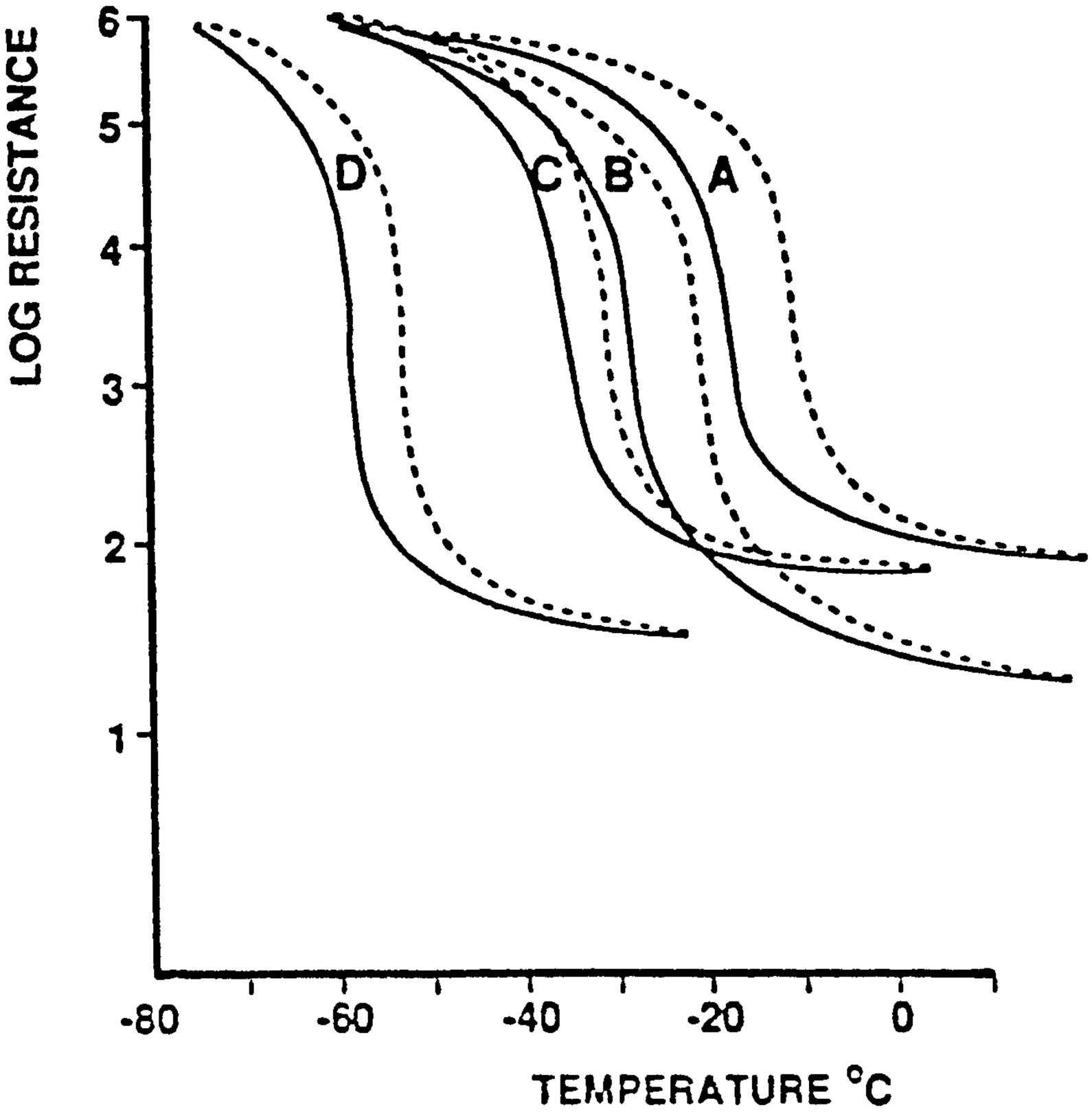


Fig 6.22 Resistivity curves for some common electrolytes. A=KCl, B=NaCl, C=$MgCl_2$, D=$CaCl_2$. The warming curves (broken lines in the figure) show the lowest eutectic temperature.

Accordingly, a device was manufactured, by Jones Bros, Bury, to the authors specifications. See fig 6.23. The device comprised a polypropylene sheath, long enough to reach into the bottom part of the vampoule, past the constriction. This outer sheath, some 45 mm in length was placed as far as possible into the vampoule and the dispensing head pushed downward. This pushed an inner dispensing needle clear of the bottom of the outer sheath by some 5 mm, as shown in fig 6.23, and activated a microswitch so that the Comp-u-pet dispensed an aliquot of thromboplastin, down the inner dispensing needle and into the bottom of the vampoule. There was no possibility of contamination of the constriction when this device was used.

Pressure was then released from the dispensing head allowing the dispensing needle to retract inside the protective outer sleeve and the entire dispensing head was then moved to the next vampoule. The dispensing head switch replaced the footswitch that was normally used to activate the compupet dispensing sequence.

Liquid nitrogen storage

Liquid nitrogen was stored in BOC Cryoproducts self-pressurising dewars of 75, 125 and 200 dm^3 nominal capacity (models EC75PC, EC125PC and EC200PC respectively, obtained from BOC Cryospeed Worsley, Manchester). Each vessel was fitted with a valved high pressure dispenser (model VHPD4) and a capacitance gauge (Model TCG1).

Fig 6.23 (opposite) Pipetting device fitted with dispensing head designed for vampoules.

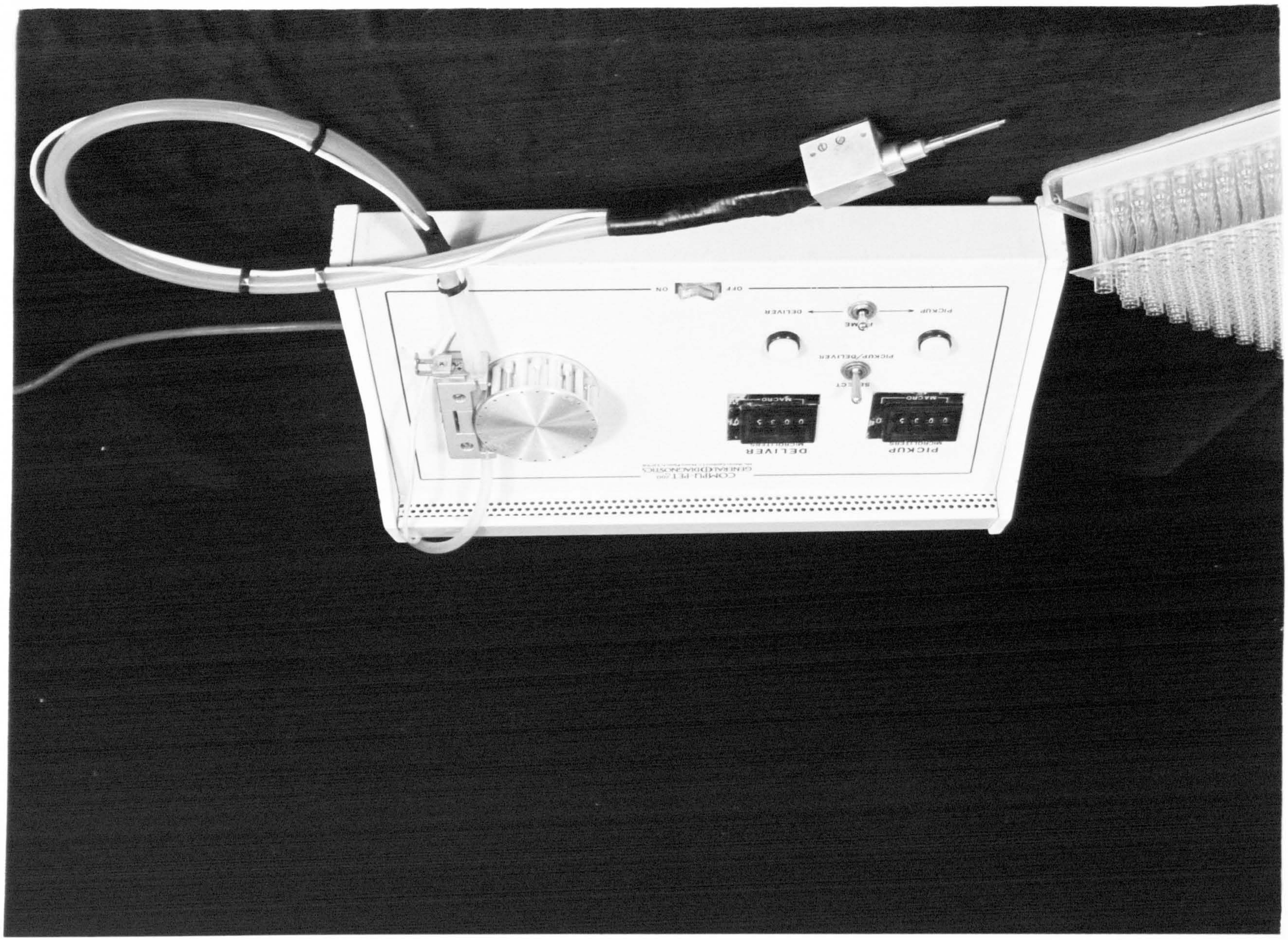

OFF
ON
PICKUP
DELIVER
PICKUP/DELIVER
SELECT
MACRO
DELIVER
PICKUP
GENERAL DIAGNOSTICS

FIG 6.23

These dewars were manufactured from stainless steel. Each had a pressure gauge and two "over-pressure" protection devices, i.e., a relief valve, which was a simple one-way spring-loaded valve which opened if the pressure exceeded one atmosphere, and, in case of failure, a "bursting" valve, in which a plastic membrane ruptured if a pressure of three atmospheres was exceeded.

Liquid nitrogen was pumped, at delivery rates up to 12 dm^3 per minute through 1/2 in nylon pipe lines. These were covered with expanded foam insulation and were fitted with pressure relief valves so that in the event of a pipeline being closed at both ends there was no possibility of pressure within it reaching dangerous levels.

6.13 THE ACTIVATED PARTIAL THROMBOPLASTIN TIME (THOMSON 1980)

Reagents

1 Owren's buffer (see Ch 6.16)

2 Calcium chloride 0.025 mol dm^{-3}

3 Kaolin (0.25 g/100 cm^3 in Owren's buffer)

4 APTT reagent. The Manchester APTT reagent is a lipid suspension which is described fully in Chapter 8.

Method

Plasma was tested as soon as possible after collection as described in Chapter 6.19. Kaolin suspension and calcium chloride were warmed, in separate test tubes, in a water bath at 37°C. Reagents were placed into a prewarmed glass test tube in the following order - 0.1 cm^3 test plasma, 0.1 cm^3 APTT reagent, 0.1 cm^3 kaolin suspension and a stop-watch started. The tube was tilted three times immediately and subsequently at approximately one minute intervals to resuspend the kaolin activator. At exactly 10 minutes 0.1 cm^3 of calcium chloride was added. The tube was tilted three times then left undisturbed for 20 seconds from the time of addition of calcium chloride. After this time the tube was tilted gently until a solid clot formed. The time from addition of calcium chloride to clot formation was the activated partial thromboplastin time. Each plasma was tested in duplicate. If duplicates differed by more than 5% then a further test was

performed, and so on, until a satisfactory agreement between tests was achieved. Normal range was 36 to 48 secs.

The APTT test is discussed in detail in Chapter 4.

6.14 THE PROTHROMBIN TIME TEST (QUICK 1935)

Reagents

1 Tissue thromboplastin reagent

2 Calcium chloride 0.025 mol dm^{-3}

Method

Plasma was tested as soon as possible after collection as described in Chapter 6.19. 0.1 cm^3 of plasma was added to a glass test tube and allowed to warm for 30 seconds in a water bath at 37°C. 0.1 cm^3 of thromboplastin reagent was added and allowed to warm for 30 seconds. Finally, 0.1 cm^3 of calcium chloride was added and a stop watch started simultaneously. The tube was tilted until a solid clot was seen to form, whereupon timing was stopped. Tests were repeated until duplicates were obtained with prothrombin times within 5% of each other. The normal range was 12 to 15 seconds.

The thromboplastin reagent is discussed in Chapter 3, above.

6.15 ASSAY OF FVIII:C AND FIX - ONE STAGE METHOD

Reagents

1 Substrate plasma was obtained from Immuno Ltd and was from a patient with a severe haemophilia A.

2 Kaolin 0.1 g/100 cm^3 in imidazole buffer.

3 Calcium chloride 0.033 mol dm^{-3}

4 Partial thromboplastin reagent - batch no. 117 of the Manchester APTT reagent.

5 Imidazole buffer - see Chapter 6.16.

6 Normal plasma pool - equal volumes of plasma from four blood group O, four blood group A and one blood group B were mixed and frozen to -80°C in 1 cm^3 aliquots. Plasma was collected from healthy adults into 0.109 mol dm^{-3} sodium citrate (9:1 v/v). The factor level was determined before use in the assay.

7 Standard reference plasmas. Plasmas with values of 100% and 20% were included.

Method

1 in 5, 1 in 10, 1 in 20, 1 in 40 and 1 in 80 dilutions (representing 200%, 100%, 50%, 25% and 12.5% activity respectively) of normal pool plasma in imidazole buffer were prepared and stored on ice prior to testing.

Mixtures of 0.1 cm^3 factor deficient substrate, 0.1 cm^3 of APTT reagent, 0.1 cm^3 of normal plasma dilution and 0.1 cm^3 kaolin suspension were prepared in glass tubes. The tests were performed in duplicate and stopwatches started on addition of kaolin. The tubes were incubated at 37°C and tilted at approximately 1 minute intervals for 10 minutes.

After 10 minutes 0.1 cm^3 of calcium chloride was added and a stopwatch started. After 20 secs the tubes were tilted and the time taken for a clot to form was recorded.

1 in 10, 1 in 20 and 1 in 40 dilutions (representing 100%, 50% and 25% activity respectively) of the "normal" and "low" plasmas in imidazole buffer were prepared and stored on ice prior to testing. These were then tested as above in the same way as the normal pool dilutions.

A graph of clotting times of the normal pool dilutions vs % concentration was prepared on double log graph paper. The clotting times of the normal and low reference plasmas were plotted on the same graph. These lines should be straight and parallel. The presence of inhibitors against any of the intrinsic clotting factors will show itself in increased factor level results in the lower dilutions causing non-parallelism.

The assay described may be used for FVIII:C or for FIX by employing relevant substrate and reference materials.

6.16 BUFFERS AND REAGENTS

Owren's buffer pH 7.35

sodium diethylbarbituric acid	5.878 g
sodium chloride	7.335 g
0.1 mol dm^{-3} HCl	215 cm^3
distilled water to 1 dm^3	

Cacodylate buffered glutaraldehyde pH 7.4

0.2 mol dm^{-3} sodium cacodylate	165 cm^3
2.5 mol dm^{-3} glutaraldehyde	100 cm^3

distilled water to 500 cm^3, adjust pH to 7.4 with 0.1 mol dm^{-3} HCl

Imidazole buffer pH 7.35

imidazole	3.4 g
sodium chloride	5.85 g
distilled water	500 cm^3
0.1 mol dm^{-3} HCl	186 cm^3
distilled water to 1 dm^3	

The following reagents were obtained from Sigma Chemical Co., Fancy Road, Poole, Dorset.

cerebrosides

cholesterol

cholesterol esters

galactosyl diglyceride

monoglycerides

1,2-diglycerides

1,3-diglycerides

triglycerides

free fatty acids

sulphatides

sphingomyelin

phosphatidic acid

phosphatidyl choline

lyso-phosphatidyl choline

phosphatidyl ethanolamine

diphosphatidyl glycerol

phosphatidl inositol

phosphatidyl serine

The following was obtained from ICN Biomedicals Ltd, Castle Street, High Wycombe, Bucks

1,1,3,3-tetraethoxypropane

The following were obtained from BOC Ltd, Worsley Road, Worsley, Manchester.

liquid nitrogen

hydrogen gas, high purity

nitrogen gas, high purity

compressed air, industrial grade

The following was obtained from Distillers CO_2 Co. Ltd, 15 Pollard St East, Manchester.

CO_2 liquid, in siphon cylinders

The following were obtained from Alltech UK, Units 6-7, Kellet Road Industrial Estate, Carnforth, Lancs.

fatty acid methyl esters, saturated from 12:0 to 22:0 and unsaturated from 14:1 to 24:1.

The following was obtained from Supelchem, Radley and Co. Ltd, Louden Road, Sawbridgeworth, Herts.

phosphatidyl glycerol

The following were obtained from EM Scope Laboratories, Kingsnorth Technology Park, Wooton Road, Ashford, Kent.

propylene oxide

sodium cacodylate

osmium tetroxide

agarose

The following were obtained from BDH Ltd, Broom Road, Poole, Dorset.

benzene

chloroform

acetone

diethyl ether

hexane

lutidine

methanol

Schiff's reagent

sulphuric acid

phosphoric acid

acetic acid

perchloric acid

ammonium molybdate

ammonium thiocyanate

ascorbic acid

calcium chloride

cupric acetate

ferric chloride hexahydrate

imidazole

kaolin, light

magnesium chloride

molecular sieve, type 4A

ninhydrin

phosphorus pentoxide

potassium chloride

potassium dihydrogen phosphate

sodium chloride

sodium diethylbarbituric acid

sodium hydroxide

sodium periodate

sulphur dioxdie

thiobarbituric acid

Extran 300

6.17 pH MEASUREMENT

pH measurements were made using a Philips PW 9409 pH meter, fitted with an Amagrass E1 3509 combined glass reference electrode (Whatman Labsales, Maidstone). The instrument was calibrated by the use of standard buffer solutions in the range pH 5.0 to pH 10.0, obtained from BDH, Poole.

6.18 MICROBIOLOGY TESTING

Colony counts of microorganisms in thromboplastin were performed by the Microbiology Laboratory, Withington Hospital. 1 cm^3 of thromboplastin was added to 19 cm^3 of cooling melted nutrient agar, shaken and poured into a sterile Petri dish. When the agar had set, the plate was incubated at 32^oC for 48 hours and a count of colonies of microorganisms was made. Thromboplastin preparations containing more than 50 colonies cm^{-3} were discarded.

The Public Health Laboratory at Withington Hospital tested both thromboplastin reagents and human plasma donations for the presence of hepatitis virus and, when a test became available, HIV. The hepatitis testing was based on a radioimmunoassay technique for hepatitis B surface antigen while testing for HIV employed a competitive ELISA method.

6.19 BIOLOGICAL REAGENTS

Preparation of tissue thromboplastin and partial thromboplastin reagents and freeze-drying of the former is described fully in the experimental section.

The other biological reagents used were plasmas, employed in degradation studies and tests of procoagulant activities of thromboplastin and partial thromboplastin reagents.

Plasmas were collected, prepared for freeze-drying, and freeze-dried as already described (Stevenson 1978). Following lyophilisation, 28 vials of each batch were tested for inter-vial variation by measuring the prothrombin time of their contents. The minimum sample of vials was calculated so that the possibility of falsely accepting a batch was kept as low as possible (Stevenson 1978).

Fresh normal plasma was collected from healthy volunteers, by venepuncture, into 0.13 mol dm^{-3} sodium citrate (9:1 v/v) and centrifuged immediately at 2000 x g for 20 minutes at $4^{o}C$, to obtain platlet-poor plasma. When platelet-free plasma was required, for example in the experiments described in Chapter 8, plasma was filtered through a Minisart NML cellulose acetate membrane (Sartorius Instruments, Sutton, Surrey) at pore size 0.2 u. Filtering was not an efficient method when large volumes of plasma were to be processed. In this case, the plasma was centrifuged at 40,000 x g for 30 minutes at $4^{o}C$, and dispensed into suitable

containers (Sarstedt Ltd, Leicester) for storage in liquid nitrogen or, alternatively, for freeze-drying, as previously described (Stevenson 1978).

6.20 MALONDIALDEHYDE ASSAY (BASED ON METHOD OF DEKONING AND SILK 1963)

The method utilizes the capacity of malondialdehyde (MDA) to react with thiobarbituric acid to give a characteristic colour.

Thiobarbituric acid reagent (TBA) - 800 mg TBA was dissolved in 10 cm^3 of 2.5mol dm^{-3} NaOH and 50 cm^3 water was added. The pH was adjusted to 7.4 by the addition of concentrated perchloric acid, and the volume brought to 100 cm^3 with distilled water. 50 cm^3 of 7% perchloric acid was added and the reagent stored in a glass-stoppered bottle at room temperature.

Assay of MDA - 2 cm^3 of test solution was placed in a test tube. 2 cm^3 of TBA was added, mixed on a vortex mixer, stoppered and heated at 100^{o}C for 15 minutes. The tubes were then cooled and centrifuged at 12000 x g for 10 minutes. The absorbance of the supernatant was read at 532 nm.

A standard curve was prepared, using tetraethoxypropane which yields one mole of MDA per mole. Concentrations of 2, 4, 6, 8 and 10 nmoles of tetraethoxypropane were used to prepare the curve. A standard curve of MDA concentration against absorbance is shown in fig 6.24..

Sinnhuber et al (1958) suggested that the red compound formed in the thiobarbituric acid test was probably a 2:1 adduct of thiobarbituric acid and malondialdehyde. This was confirmed by Nair

and Turner (1984) who believed that formation of the 2:1 adduct probably is initiated by nucleophilic attack involving carbon-5 of thiobarbituric acid onto carbon-1 of malondialdehyde followed by dehydration and similar subsequent reaction of the intermediate 1:1 adduct with a second molecule of thiobarbituric acid.

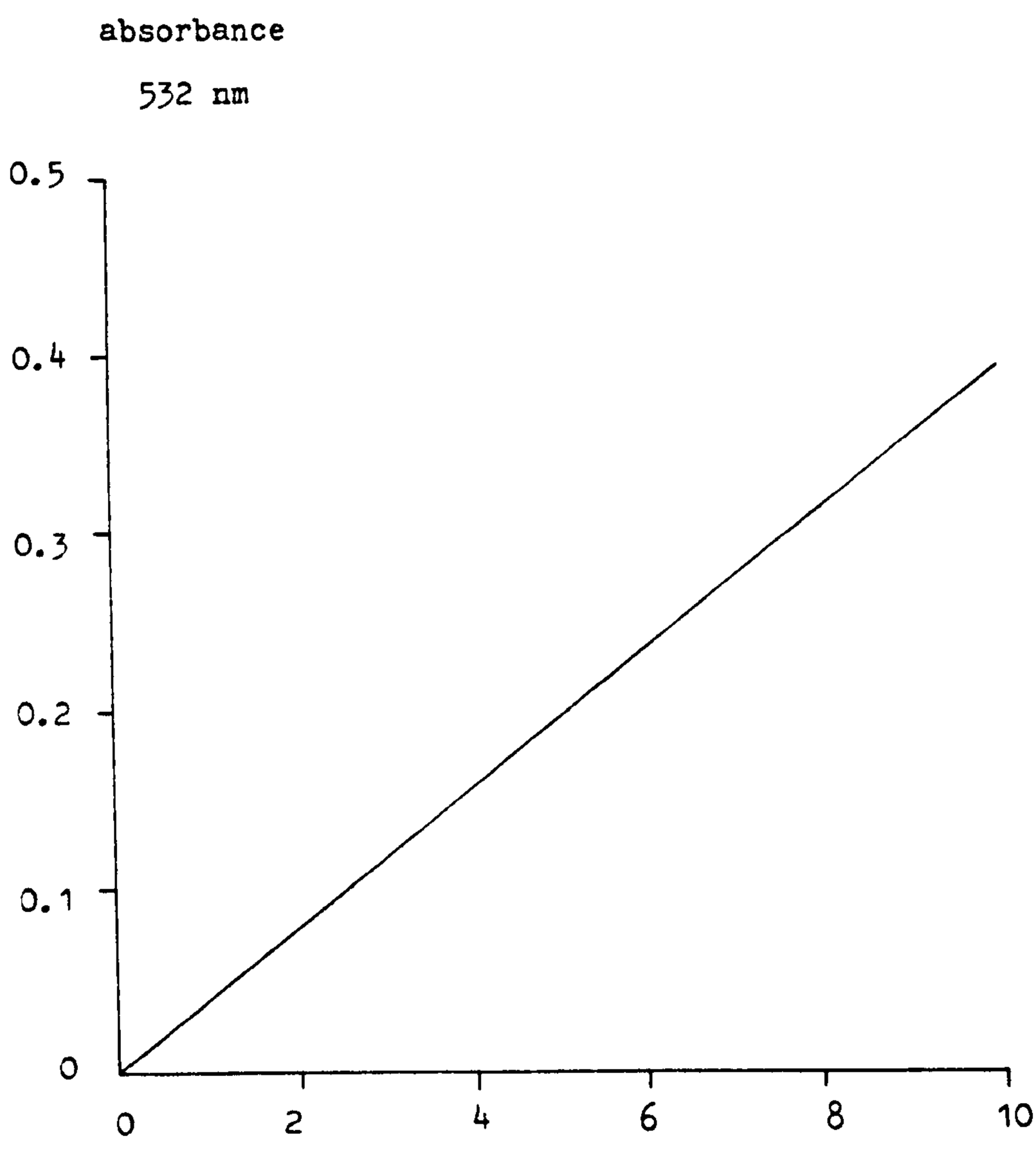


Fig. 6.24 Standard curve for malondialdehyde.

6.21 STATISTICAL METHODS

1 Analysis of variance (ANOVA).

In performing ANOVA, the null hypothesis, H_o, is that all our samples have been drawn from the same population and that the variability caused by the "within sample" variability is the same as that caused by the "between sample" variability. H_o is rejected if these are not the same. The principle of the technique is to compute the separate variances due to the variability within the samples and that between the samples. The significance of any departure from a ratio of 1 for these variabilities is tested by means of the F test. The technique was applied to results of changing relative concentrations of fatty acids in stored thromboplastin, to data on the effect of reducing condenser-temperature on residual moisture in freeze-drying, and to analysis of possible intrinsic variation in the freeze-drying machinery. The analysis was performed as described by Scheffe (1959) using a computer program known as GLIM 3.77, run on the University of Manchester Computing Centre's facilities into which the UK Reference Laboratory is connected via a system called "Systime".

GLIM 3.77 was produced by the Royal Statistical Society of London, which holds the copyright (1985).

2 Tukey test (Fowler and Cohen, 1986)

The analysis of variance does not provide a complete answer to the search for statistically significant variation between the means

of the sample groups tested, i.e. it may be that all possible combinations of pairs are different from each other or maybe just one is different from all the others. The Tukey test is performed to show which is the case. A test statistic T is computed according to the formula

$$T = (q) \times \sqrt{\frac{\text{within variance}}{n}}$$

where n is the number of sampling units in each sample. The "within variance" is obtained from the ANOVA calculation and the value of q is found from a table of the probability distribution of q for varying numbers of samples and degrees of freedom. If any of the mean differences in the data exceed the value of T, then they are significantly different at p = 0.05.

3 Linear regression analysis was performed, to obtain correlation coefficients, as described by Bailey (1968). In Chapter 7 the technique was employed to determine the significance of the relationship between loss of procoagulant activity of stored tissue thromboplastin reagents and changing concentrations of some lipids and malondialdehyde. A program, written in BASIC, was used for this method and was run on the Spectra Physics SP4100 Computing Integrator described in Chapter 6.4. Significance was assessed by referring to the Biometrika tables (table 13.Percentage points for the distribution of the correlation coefficient).

4 Normal range determination (Wetherill 1981).

In Chapter 8, normal ranges were calculated for a number of partial thromboplastin reagents. The distribution of the data proved to be non-Gaussian so a transformation was undertaken, according to Box and Cox (1964). Each normal clotting time (x) was transformed to $x^{(\lambda)}$ using the formula $x^{(\lambda)} = (x^{\lambda} - 1)/\lambda$.

The value for λ chosen was the one for which the distribution of $x^{(\lambda)}$ most resembled a Gaussian distribution. The method for finding the best value for λ for a particular reagent was to calculate the function $L(\lambda)$ for a range of λ and to select the value of λ which gave the highest value of $L(\lambda)$. This function was calculated by

$$L(\lambda) = \frac{-n}{2} \log_e (SD\lambda)^2 + (\lambda - 1) \sum_{i=1}^{n} \log_e x_i^{(\lambda)}$$

where n is the number of normal values and $SD\lambda$ is the sample standard deviation of the transformed values. A suitable range of to use was -3 to +3 in steps of 0.25. When $\lambda = 0$ the transformation was $x^{(\lambda)} = \log_e x$. The normal range for transformed data was given by

$$[\bar{x}^{(\lambda)} - SD\lambda t_{(n-1)}, \bar{x}^{(\lambda)} + SD\lambda t_{(n-1)}]$$

where $t_{(n-1)}$ may be obtained from standard statistical tables (Pearson and Hartley 1976) $\bar{x}^{(\lambda)}$ = mean of the transformed values. In order to present the above range in seconds, the transformation needs to be reversed: if a is the lower limit of the range, then the lower limit of the normal range, in seconds, is

$(a^{\lambda} + 1)^{1/\lambda}$ or e^a if $\lambda = 0$

Similarly, if b is the upper limit of the above range, then the upper limit in seconds is

$$(b^{\lambda} + 1)^{\frac{1}{\lambda}} \quad \text{or } e^{b} \text{ if } \lambda = 0$$

These calculations were performed on the University of Manchester Computing Centre's computer via the "Systime" link from the UK Reference Laboratory. The transformation used i.e. the value of λ for the reagents was as shown in table 6.15.

TABLE 6.15 TRANSFORMATION ($y = x^{\lambda}$)

reagent	λ
Actin	2
Actin FS	1.75
Activated Thrombofax	2
Automated APTT	2.5
Manchester APTT	2.5

5 Ranking of reagent performance (Siegel 1956).

The performance of the reagents examined in Chapter 8 was correlated with particle size, electrophoretic mobility, and a variety of lipid compositional data. Using Spearman's rank correlation test (Spearman 1904), two sets of measurements could be compared. Differences between rank order were calculated, squared, and the correlation coefficient calculated from the formula

$$r_s = 1 - \frac{6 \sum d^2}{n(n^2-1)}$$

where d is the difference between ranks for each reagent and n is the number of ranks. The range of r_s is from -1 (complete

discordance) to +1 (complete concordance). A program, written in BASIC, was employed for this test and was run on the Spectra Physics SP4100 computing integrator.

Following the establishment of correlations between clotting performance and physical and chemical composition of the reagents, they were ranked according to the formula

rank % = (rank sum/highest possible rank sum) x 100

The result may be seen in fig 8.10.

6 t test (Swinscow 1976)

The paired t test was used in Chapter 9.3 to determine whether there was a significant difference between two sets of residual moisture data obtained when a batch of tissue thromboplastin reagent was lyophilised using either a P_2O_5 trap during the desorption stage or a liquid nitrogen-cooled trap. The calculation was performed using a program, written in BASIC, that ran on the Spectra Physics SP4100 computing integrator.

CHAPTER 7 EXPERIMENTS ON TISSUE THROMBOPLASTIN

The preparation and use of the British Comparative Thromboplastin (BCT) have been presented in Chapter 3. The experiments described in this chapter have been designed with the following aims:

1 to reveal the nature of the BCT by electron microscopy of fresh and aged preparations and

2 to show whether the loss of procoagulant activity on ageing correlates with changes in the lipid moiety of the material.

7.1 TO INVESTIGATE THE MORPHOLOGY OF BRITISH COMPARATIVE THROMBOPLASTIN (BCT)

Aim - to examine the ultrastructure of the BCT by electron microscopy.

Procedure - an aliquot of a single, freshly prepared batch of BCT was subjected to electron microscopical examination. The material was fixed and prepared for microscopy as described in Chapter 6.8, and examined in an AEI EM 601 transmission electron microscope.

Results - Fig 7.1 shows an electron micrograph of BCT at a magnification of 12,500 times. This field was typical of those examined. Myelin sheaths (MS) are present in abundance, some still surrounding debris from nerve cells. Two oligodendroglial cell nuclei (N) are to be seen and a variety of membranous fragments and occasional vesicles make up the remainder. There is no evidence of sub-cellular organelles, other than the nuclei already mentioned.

Discussion - The method for extraction of human brain with phenol-saline, to produce a thromboplastin reagent, was originally described by Poller (1970) and later by the present author (Stevenson 1978). The most striking features are the quantity of myelin sheaths in the reagent and the degree of order in their structure (see fig 7.1). While there are some fragments of myelin and some membranous vesicles, it is clear that the bulk of the membranous material in BCT is in a highly ordered state. This batch

Fig 7.1 (opposite) Electron micrograph of a fresh preparation of BCT. Magnification X 12 500.

N=nucleus. MS=myelin sheath. Scale bar is 500nm.

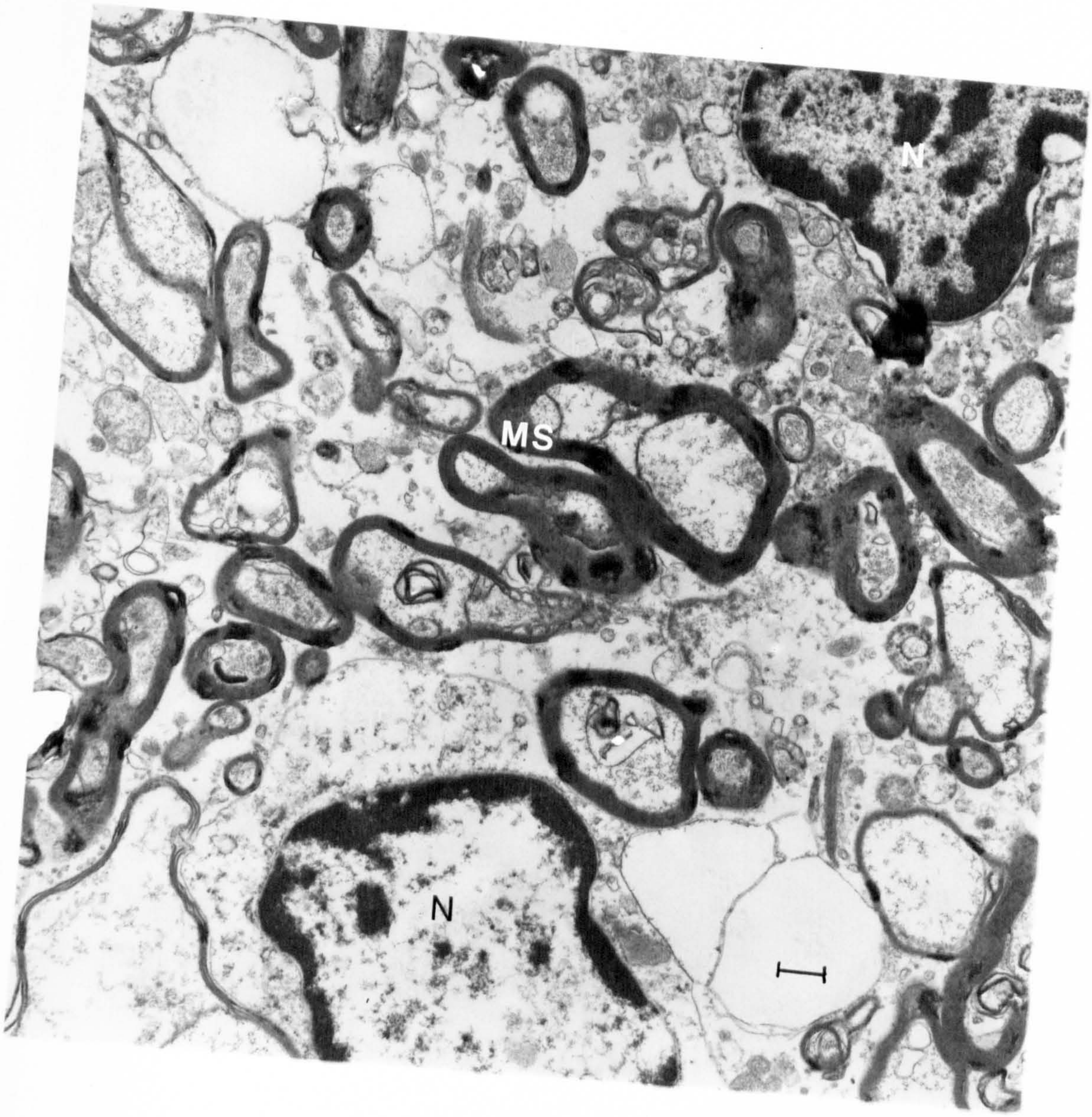


FIG 7.1

of BCT had been prepared four weeks prior to the micrograph being taken. The procoagulant activity of this batch was optimal (see Chapter 7.3) which had been confirmed by successful external multicentre monitoring (see Chapter 3) designed to monitor its performance in the prothrombin time test. Two other groups of workers have published morphological studies of tissue thromboplastin; Hvatum et al (1969) and Hasegawa et al (1977). In the first of these, the human brain thromboplastin reagent studied had been subjected to a purification sequence that involved centrifugation, gel filtration and solubilisation with sodium deoxycholate followed by dialysis to remove the detergent and recover thromboplastic activity. With hindsight it is possible to say that this treatment will have formed liposomes (see Chapter 8) and that all that was observed was a pattern of concentrically arranged membranous structures, typical of liposomes. Indeed, the authors stated that the structures resembled the smectic mesophases (liposomes) described by Bangham and Horne (1964) and also observed some resemblance to myelin sheaths, a belief not shared by the present author.

Hasegawa et al (1977) used a commercially manufactured thromboplastin reagent extracted from rabbit lungs. Concentrically arranged membranous structures were observed which, once again, did not resemble myelin. Neither Hvatum and colleagues nor Hasegawa's group observed any subcellular structures in their preparations, presumably a consequence of the extraction methods used.

BCT was a suspension of human brains macerated in phenol-saline and had no detergent added and no gel filtration steps in its preparation. The resulting suspension, therefore, was comprised of myelin sheaths in a wide range of size, cell nuclei and a great deal of membranous debris.

The possibility that changes in morphology may be related to chemical changes in the reagent, e.g. oxidation of fatty acids, and to loss of procoagulant activity is discussed in the next section.

Conclusion - BCT was found to be comprised of myelin sheaths with cell nuclei and various membranous debris in attendance. No subcellular structures other than nuclei survived the extraction process intact.

7.2 OBSERVATIONS ON THE MORPHOLOGY OF TISSUE THROMBOPLASTIN REAGENT STORED AT 4°C

Aim - to show whether there is a discernable change in the morphology of myelin figures in BCT preparations during long-term storage at 4°C.

Procedure - three BCT preparations were sampled and prepared for electron microscopy, as described in Chapter 6.8. These were batches numbered 30, 16 and 1 which were respectively 1, 51 and 102 months old at the time of testing (see table 7.1).

Results - electron micrographs of BCT reagent myelin figures are shown in figures 7.2, 7.3 and 7.4 at magnifications of 32,000, 40,000 and 40,000 times respectively. Fig 7.2 shows a typical myelin sheath from the fresh extract, BCT batch 30. There is a highly ordered structure to be seen in the micrograph, with the membranes tightly associated and with little sign of degradation. A typical myelin sheath from BCT batch 16, which had been stored for some 51 months may be seen in fig 7.3. No subcellular structures of any kind were recognizable in this preparation, the myelin figures being the only structures which had maintained any form. It is noticeable that the membrane lamellae are less ordered than in the fresh preparation and are loosening and opening.

A myelin figure from BCT 1, stored for 102 months, is illustrated in fig 7.4. Further degradation than that observed in fig 7.3 is evidenced by loss of the regular ordered form of the

Fig 7.2 (opposite) Electron micrograph of a myelin sheath from a fresh BCT preparation. Magnification X 32 000. Scale bar is 500nm.

Fig 7.3 (p209) Electron micrograph of a myelin sheath from a BCT preparation stored at 4°C for 51 months. Magnification X 40 000. Scale bar is 500nm.

Fig 7.4 (p210) Electron micrograph of a myelin sheath from a BCT preparation stored at 4°C for 102 months. Magnification X 40 000. Scale bar is 500nm.

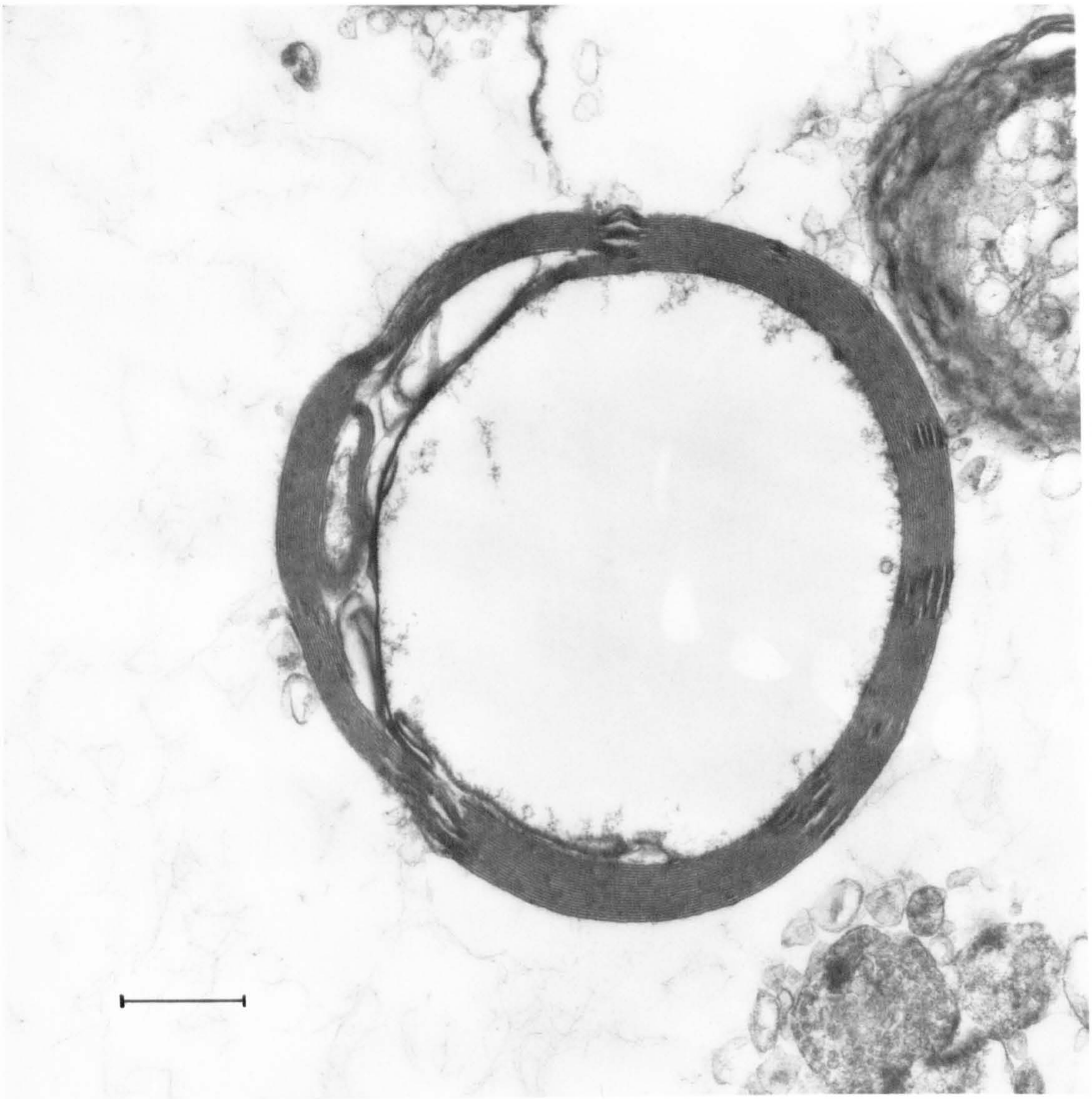


Fig 7.2

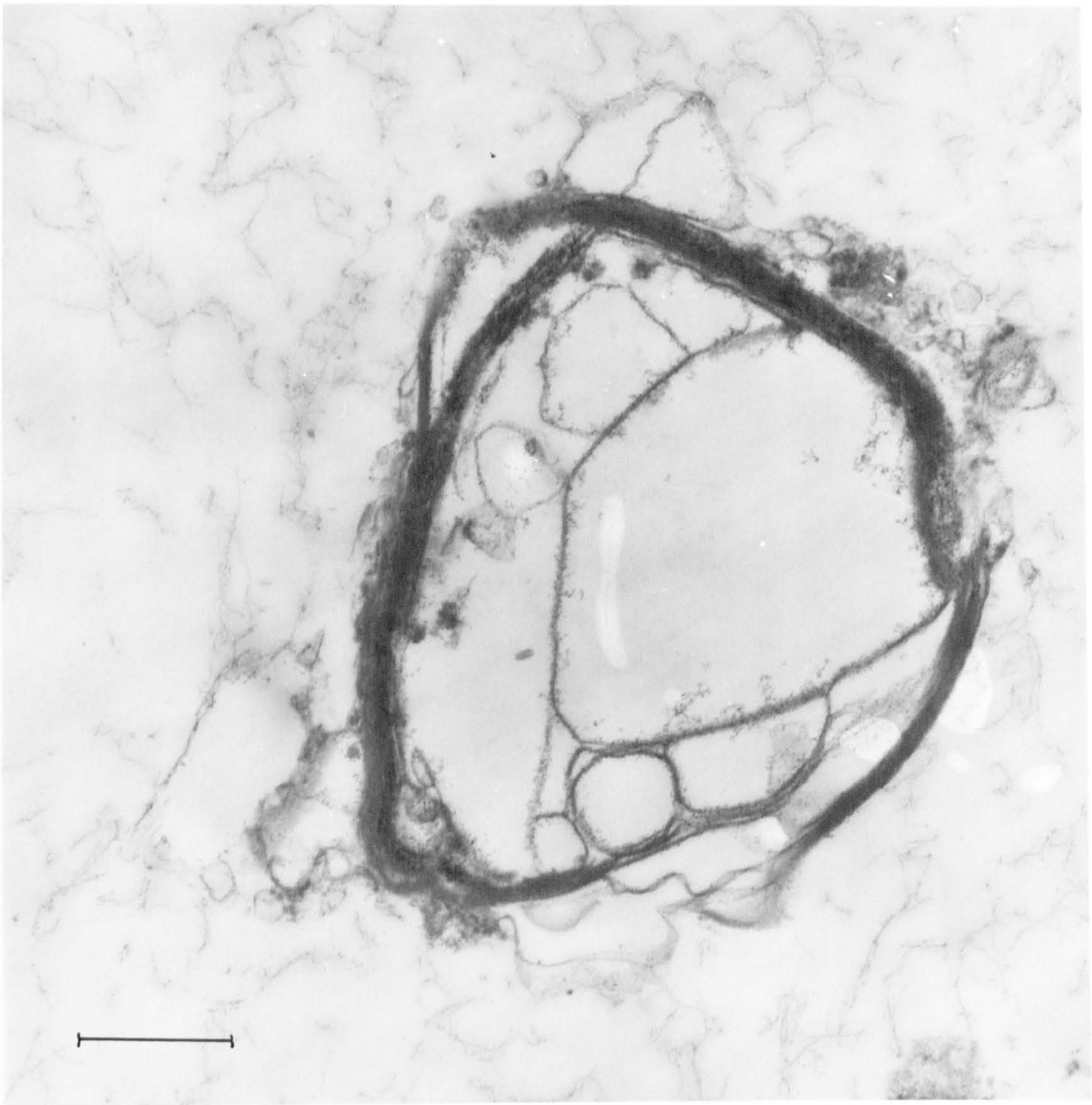


Fig 7.3

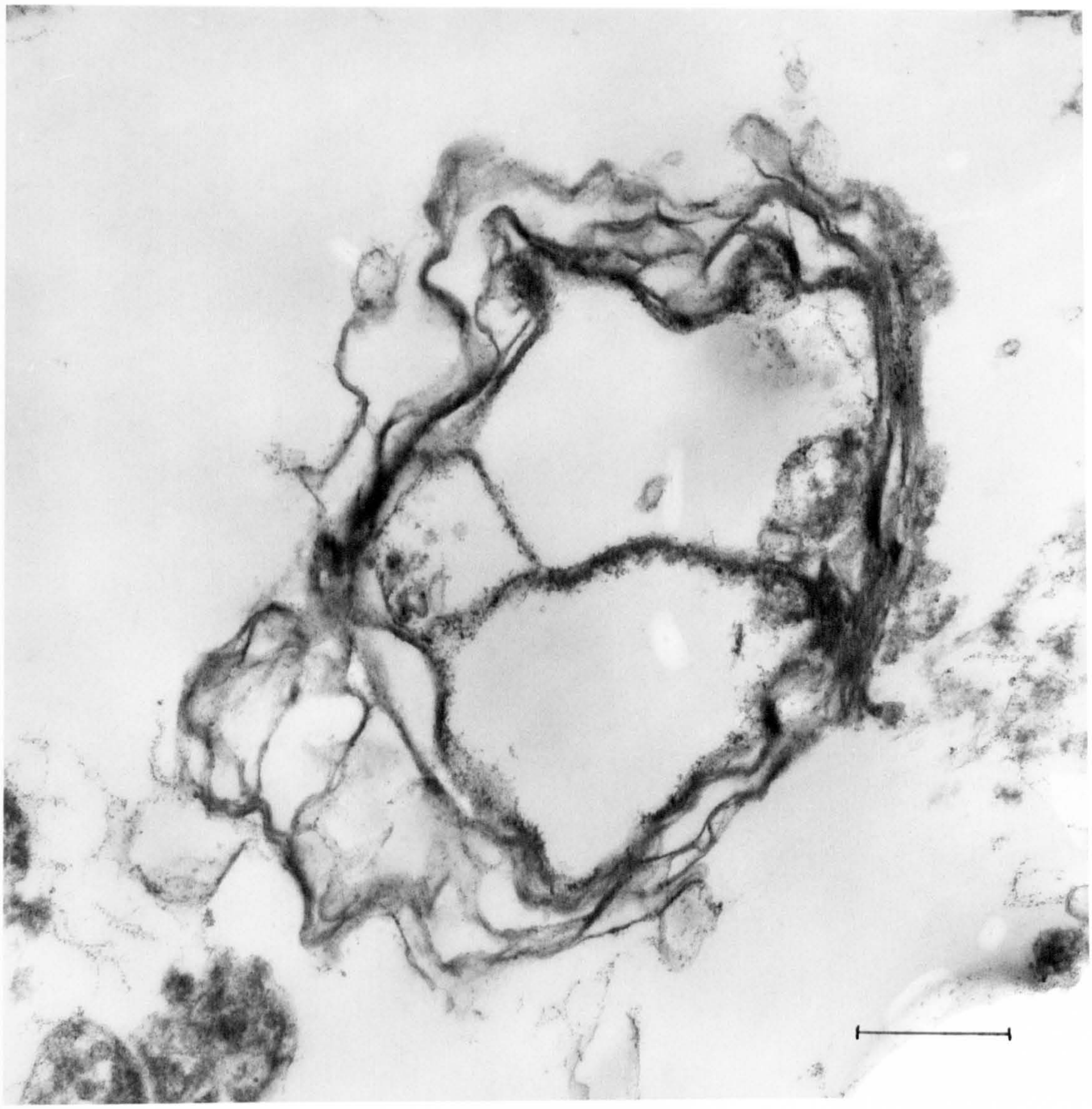


FIG 7.4

fresh preparation. Remarkably, even after this extended storage time, there are still segments of the sheath where the lamellae are tightly associated. These observations were made during examination of large numbers of fields in the electron microscope and the figures show typical features seen.

Discussion - the highly ordered structure of myelin sheath membrane has been commented on previously (e.g. Boggs and Moscarello 1978). It is this feature that is the most striking in this series of electron micrographs (figs 7.1 to 7.4), which reveal a clear trend towards loss of order in the myelin figures with time of storage. Changes in the lipid composition of the reagents, with time are considered in the next section.

Conclusion - the major change that is to be seen in tissue thromboplastin preparations undergoing long term storage at $4^{o}C$, is the degradation of the highly ordered arrangement of membranes that make up the myelin sheath. The oldest preparation examined, BCT 1, had few recognizable myelin figures remaining.

7.3 THE LIPID COMPOSITION OF NEW AND AGED BATCHES OF THE BCT AND ITS RELATIONSHIP TO PROCOAGULANT ACTIVITY

Aim - to determine the lipid composition of BCT batches, both fresh and stored at $4^{o}C$ for up to 102 months and to examine whether there is any correlation between change in lipid composition with time of storage and loss of procoagulant activity.

Procedure - lipids were extracted from the batches of BCT listed in Table 7.1. Also shown in the table are the monitoring dates of each batch and their age at testing. The methyl esters of the fatty acids were derived as described in Chapter 6.4. The lipid class composition was determined by thin-layer chromatography and densitometry as described in Chapter 6.1. Fatty acid methyl esters were separated and quantified by gas chromatography on capillary columns as described in Chapter 6.4. Malondialdehyde (mda) was determined as described in Chapter 6.20. Two statistical methods were employed. Firstly, analysis of variance was used to show whether changes in fatty acid composition with time were significant and, secondly, to determine whether a change of concentration of a particular lipid class, or mda, with time was significant, the correlation coefficient was calculated and the level of significance obtained by consulting tables (see Chapter 6.21).

For measurement of procoagulant activity, three lyophilised plasmas were prepared, the first from a normal healthy donor, the second from a patient stabilized on oral anticoagulants and the third a pool of plasma from a colony of beagle dogs congenitally

deficient in factor VII. The prothrombin time of each plasma was measured using each test batch of BCT in the one-stage prothrombin time test. Preparation of the plasmas is described in Chapter 6.19 and the prothrombin time test in Chapter 6.14. Loss of procoagulant activity (as shown by lengthening prothrombin time of the normal plasma) was correlated against change in concentration of some lipid components and mda and the level of significance of the correlation coefficient obtained from tables. This was to determine which component in the BCT correlated best with loss of procoagulant activity.

TABLE 7.1 BATCHES OF BCT USED IN THE STUDY - MONITORING DATE AND AGE AT TIME OF TESTING

BCT No.	Monitoring date	Age at testing (months)
1	November 1969	102
4	November 1970	90
8	January 1972	76
12	March 1973	62
16	February 1974	51
20	June 1975	35
23	March 1976	26
26	February 1977	15
29	January 1978	4
30	April 1978	1

Results - the lipid composition of BCT (batch 30) is given in Table 7.2

TABLE 7.2 LIPID COMPOSITION OF BCT 30 (g dm^{-3})

PC	PE	SPH	PS	PI	FFA	Chol	MG	DG	TG	Sul	Cerb
1.84	2.10	0.87	1.02	0.16	0.05	0.24	0.18	0.36	0.36	0.12	0.48

The decreasing concentration of those phospholipid classes which changed significantly on storage are illustrated (fig 7.5). The increasing FFA concentration is also shown. Components which did not change significantly are not presented. The increasing concentration of mda with time of storage is the subject of fig 7.6. Correlation coefficients, obtained when changing concentrations of PC, PE, PS, PI, FFA and MDA were correlated with time, and listed in Table 7.3. These were very highly significant.

TABLE 7.3 CORRELATION COEFFICIENTS AND SIGNIFICANCE LEVELS OBTAINED BY CORRELATING CHANGING CONCENTRATIONS OF LIPIDS AND MDA IN STORED BCT WITH TIME OF STORAGE

Component	Correlation coefficient	Significance level
PC	-0.974	$p < 0.001$
PE	-0.915	$p < 0.001$
PS	-0.986	$p < 0.001$
PI	-0.931	$p < 0.001$
FFA	0.976	$p < 0.001$
MDA	0.956	$p < 0.001$

The loss of procoagulant activity according to time of storage of BCT is given in fig 7.7. Prothrombin times of lyophilised, calibrated plasmas lengthen with storage time.

When the decreasing procoagulant activity of a normal plasma is correlated against changing concentrations of lipids in fig 7.5, and mda (fig 7.6) some highly significant correlations were observed (Table 7.4).

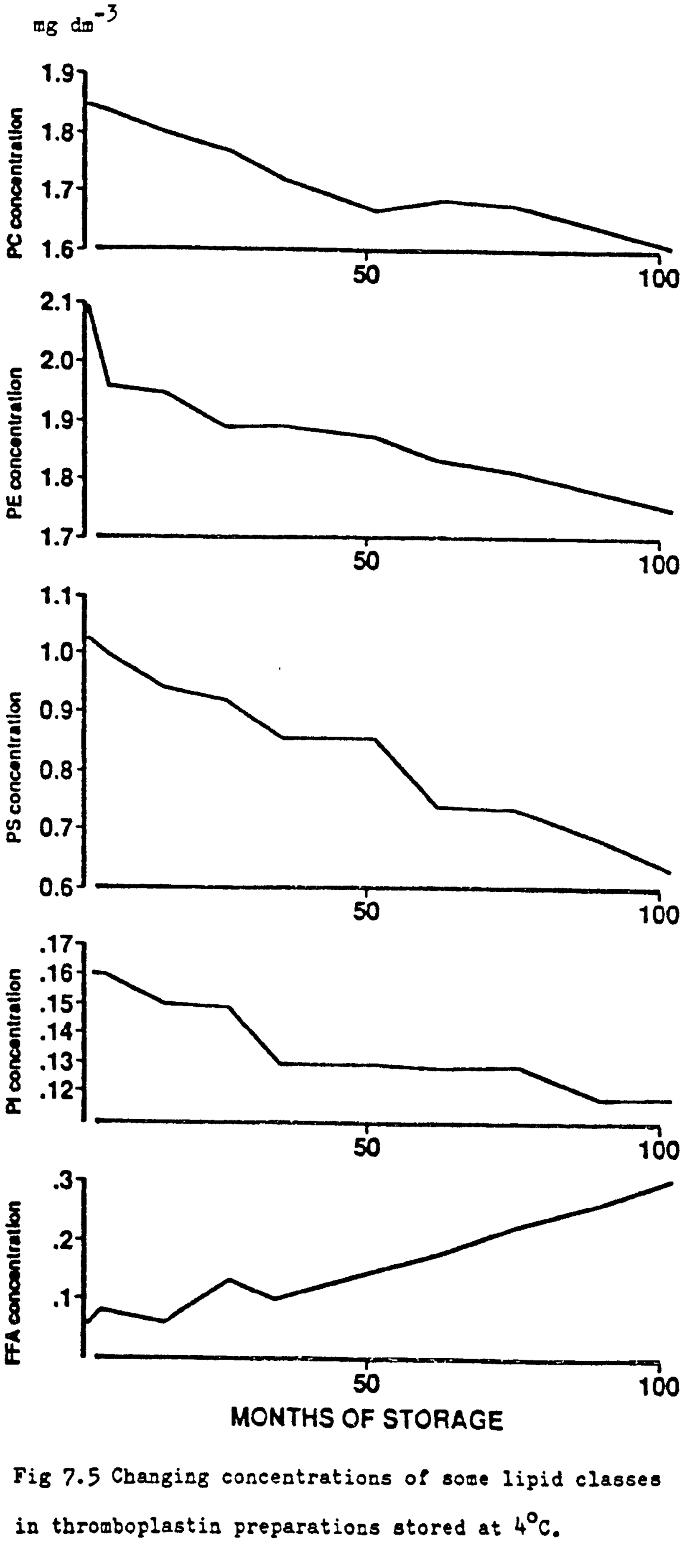


Fig 7.5 Changing concentrations of some lipid classes in thromboplastin preparations stored at 4°C.

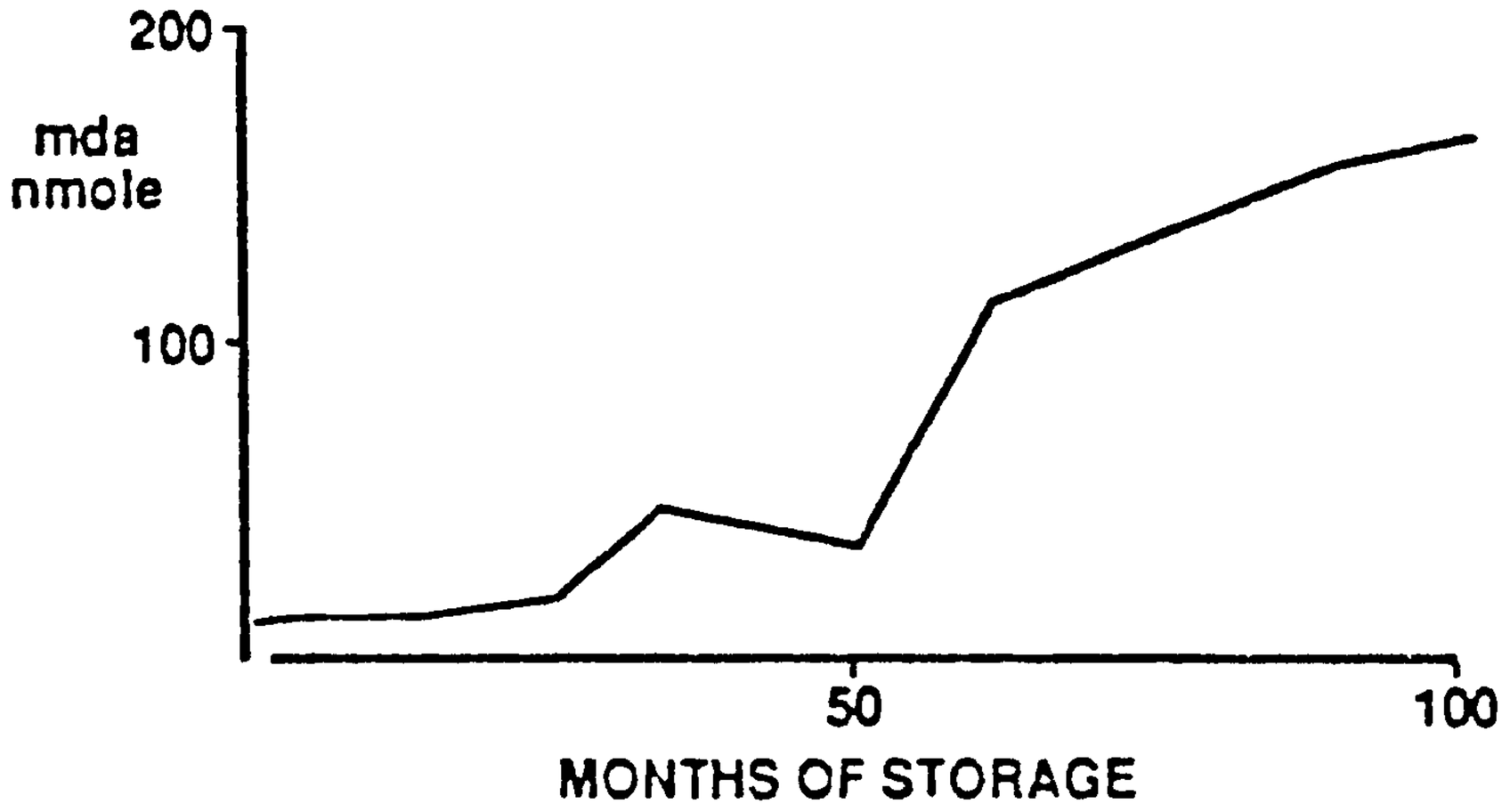


Fig 7.6 Increase in malondialdehyde concentration with time of storage of BCT.

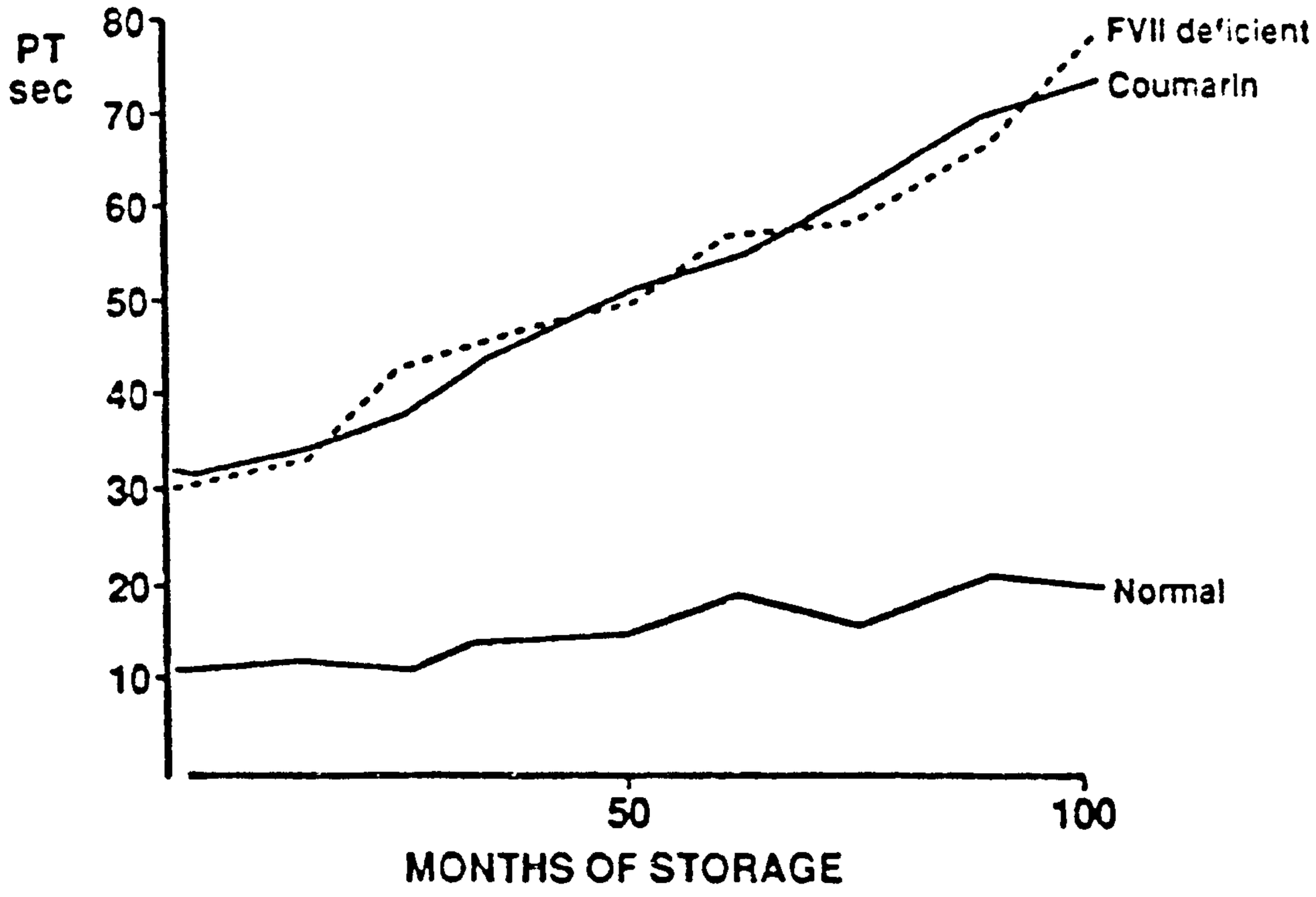


Fig 7.7 Loss of procoagulant activity with time of storage.

TABLE 7.4 CORRELATION COEFFICIENTS AND SIGNIFICANCE LEVELS OBTAINED BY CORRELATING CHANGING CONCENTRATIONS OF LIPIDS AND MDA IN STORED BCT WITH DECREASE IN PROCOAGULANT ACTIVITY

Component	Correlation coefficient	Significance level
PC	-0.8954	$p < 0.001$
PE	-0.8223	$p < 0.005$
PS	-0.9501	$p < 0.001$
PI	-0.9041	$p < 0.001$
FFA	0.8896	$p < 0.001$
MDA	0.9388	$p < 0.001$

Only the correlation of PE concentration with decreasing procoagulant activity falls below the very highly significant level of $p < 0.001$.

Table 7.5 shows the distribution of the fatty acids of tissue thromboplastin and the change in their distribution with time of storage. A summary of the results when analysis of variance was applied to the data is given in table 7.6. An upward trend indicates that a particular component is increasing in relative concentration with age, while a downward trend is the reverse. The ratio of unsaturated to saturated fatty acids (USFAR) decreases as the time of storage increases, due to the loss of unsaturated fatty acids e.g. 22:6, 24:1, 20:4 and 18:2.

TABLE 7.5 DISTRIBUTION OF THE FATTY ACIDS IN STORED BCT. CONCENTRATION EXPRESSED AS % OF TOTAL

Fatty acid	BCT batch No. 30	29	26	23	20	16	12	8	4	1
12:0	.48	.45	.41	-	.38	.25	.32	.26	.25	.23
14:0	.82	.80	.83	-	.78	.85	.80	.73	.76	.70
16:0	17.57	17.01	17.10	17.99	18.41	20.89	20.87	20.91	21.21	20.94
18:0	27.72	27.91	27.83	28.83	28.86	28.96	29.41	30.04	31.74	32.18
20:0	.35	.30	.23	-	.21	-	.30	.24	.25	.32
22:0	.20	-	.15	-	-	-	.10	-	-	-
14:1	.24	.18	.21	-	.10	.13	-	.11	.07	-
16:1	1.55	1.48	1.30	1.18	1.17	1.10	.98	.99	.91	.87
18:1	27.84	27.61	26.85	25.60	28.13	31.46	29.89	28.7	28.52	28.27
18:2	.96	.87	.80	.53	.67	.64	.65	.63	.64	.63
18:3ω6,9,12	.60	.55	.31	.12	.14	.17	.14	.10	.12	.08
18:3ω9,12,15	.60	.52	.40	.25	.38	.38	.36	.37	.33	.35
20:1	2.12	3.1	3.34	4.85	3.90	3.78	3.60	3.70	3.21	2.60
20:2	.60	.55	.67	.84	.58	.42	.47	.45	.46	.47
20:3	-	-	.12	.53	-	-	.10	-	.21	-
20:4	8.36	8.20	8.18	8.05	5.76	5.35	5.44	5.10	5.40	5.48
22:1	.48	.30	.16	-	.16	-	.05	-	.10	.16
22:2	1.19	1.10	1.02	1.65	1.31	-	1.13	-	1.00	1.03
22:6	9.44	9.39	8.40	8.14	6.21	5.61	5.50	5.46	4.90	4.81
24:1	1.79	1.73	1.58	1.46	1.26	1.33	1.30	1.17	.98	.87
USFAR	1.18	1.20	1.15	1.14	1.02	0.99	0.96	0.90	0.86	0.84

TABLE 7.6 SUMMARY OF FINDINGS BY ANALYSIS OF VARIANCE (ANOVA)

Fatty acid	Finding by ANOVA	
12:0	significant downward trend	$p < 0.001$
14:0	significant downward trend	$p < 0.05$
16:0	significant upward trend	$p < 0.001$
18:0	significant upward trend	$p < 0.001$
20:0	no significant trend	$p > 0.05$
22:0	insufficient data	-
14:1	significant downward trend	$p < 0.05$
16:1	significant downward trend	$p < 0.001$
18:1	no significant trend	$p > 0.05$
18:2	significant downward trend	$p < 0.05$
18:3 ω 6, 9, 12	significant downward trend	$p < 0.01$
18:3 ω 9, 12, 15	no significant trend	$p > 0.05$
20:1	no significant trend	$p > 0.05$
20:2	no significant trend	$p > 0.05$
20:3	insufficient data	-
20:4	significant downward trend	$p < 0.01$
22:1	no significant trend	$p > 0.05$
22:2	no significant trend	$p > 0.05$
22:6	significant downward trend	$p < 0.001$
24:1	significant downward trend	$p < 0.001$
USFAR	significant downward trend	$p < 0.001$

Discussion - Monitoring of the first batch of BCT (No 1) occurred in November 1969. Aliquots of this batch, and of successive batches, have been stored at $4^{o}C$ since their manufacture. The last batch, number 30, was monitored in April 1978. The liquid BCT was then replaced by a lyophilised preparation, batch 76/005 (Stevenson 1978).

BCT preparations stored at $4^{o}C$ for several years are subject to degradation. The shelf life of the liquid BCT was approximately six weeks and the reagent was never used beyond this time. Aliquots of BCT stored at $4^{o}C$ for up to 102 months provided the material for this study. The procoagulant activity of the reagent was found

to decrease with time. Coincident with this decrease was a change in the lipid composition. A reduction in some of the phospholipid classes was found to be statistically significant, as was change in concentration of some of the fatty acids. The free fatty acid component of the material increased, as did the mda level. These findings are strongly indicative of an oxidative mechanism in the stored material. The reagent is extracted from human brains which have been stripped of their blood vessels and washed free of blood, before being subjected to maceration in phenol-saline. This process causes the breaking down of cell membranes and the release of many potentially oxidative compounds from sub-cellular particles. The electron micrographs in figs 7.1 to 7.4 support the idea of a massive disruption of cell membranes and show that sub-cellular particles do not survive the extraction process. It is unlikely that the washing procedure will remove all of the haematin compounds from the brains. These are reported to be powerful catalysts for the oxidation of unsaturated lipids (Tappel 1953). Haemoglobin and cytochrome C, according to Tappel (1955), have catalytic activity of the same order of magnitude and equal to that of iron protoporphyrin. Tappel showed that the rate of oxidation of colloidal linoleate in the presence of these catalysts correlated with the square root of the catalyst concentration.

Mda concentration was measured by the thiobarbituric acid reaction (Buege and Aust 1978, DeKoning and Silk 1963). Dahle et al (1962) showed that this reaction provided a semiquantitative measure of the autoxidative splitting of fatty acids with three or more double bonds. The results, illustrated in fig 7.6 are consistent

consistent with an oxidative degradation of the stored BCT, and show an "induction period" of about 15 months of storage, during which the mda level was unchanged, followed by an increasing concentration that coincided with loss of procoagulant activity in the stored reagents. Barber and Wilbur (1959) showed that, in general, tissue homogenates from brain, liver and kidney readily undergo peroxidation while those from testis and intestine do not. Maceration of the human brain tissue and extraction into phenol saline may have destroyed the normal protective mechanisms that militate against autoxidative changes, e.g. glutathione peroxidase will not be available once generation of NADPH is ceased (Cohen and Hochstein 1961). Autoxidation of contaminating haemoglobin in the reagent may lead to formation of superoxide anion radical. This will not be removed after residual superoxide dismutase has lost its activity. Any formation of H_2O_2 may serve to generate radical species with the capacity to initiate peroxidation. Hochstein (1981) discussed the ability of haemoglobin to enhance the formation of hydroxyl radical, from H_2O_2 by acting as a Fenton reagent. Mead (1982) discussed the rate of oxidations in biological membranes and suggested that phospholipases A2 and C may be involved in cleaving oxidised fatty acids from phospholipids or in removing the diglyceride, in the case of phospholipase C. The latter could then by acylated and triglyceride formed. O'Brien (1987) also reported that fatty acid epoxide is a ten-fold better substrate for epoxide hydrolase than phospholipid epoxide.

The highly reactive nature of mda has been noted by several workers. Chio and Tappel (1969) and Tappel (1973) showed that mda

facilitates reaction of phospholipids with proteins by crosslinking, through Schiffs base formation, the amino groups of phospholipids and proteins. Nair et al (1981) showed that mda reacts rapidly at the α-amino group of amino acids to form 1:1 adducts, and Nair et al (1986) described a variety of adducts formed by such reactions. The polymerisation of the erythrocyte membrane protein, spectrin, through mda crosslinking, has been described by Rice-Evans and Hochstein (1981).

The oxidation process, therefore, could cause destruction of the membrane lipids and lead to polymerisation of proteins and interactions, through mda crosslinking, between phospholipid and protein components of the reagent resulting in the loss of procoagulant activity of the material. Loss of procoagulant activity correlated with reduction in concentration of some phospholipid components i.e. PC, PE, PS and PI and loss of some unsaturated fatty acids. An increase was noted in the concentrations of FFA and mda.

Stevenson (1978) reported this oxidative process, which has been extended in the present work to include further details of the change to the fatty acid components. It was demonstrated in the earlier study that oxidation could be retarded by the addition of antioxidant compounds such as propylgallate, nordihydroguaiaretic acid and ascorbic acid. Unfortunately such additions had an adverse effect on the procoagulant activity of tissue thromboplastin reagents.

Conclusion - The shelf life, of six weeks, assigned to the liquid BCT preparation was a safe one. Loss of procoagulant activity on long-term storage coincided with the loss of some phospholipid components, a reduction in unsaturated to saturated fatty acid ratio and an increase in free fatty acids and malondialdehyde concentration, indicative of an autoxidation process.

7.4 SUMMARY

The preparation, from human brains, of tissue thromboplastin reagents i.e. British Comparative Thromboplastin (BCT), the international reference preparation, and the Manchester Comparative Reagent (MCR), the routine working thromboplastin, ceased in December 1985. Up to this time a majority of hospitals in the UK used MCR and BCT was despatched to over 70 countries overseas. Three batches of lyophilised BCT prepared prior to this date are currently still serving as Reference Preparations (see Chapter 3) of the WHO, EEC and ICSH. It appears unlikely however, that further human brain-derived thromboplastin reagents will be issued for routine or reference purposes on account of the possibility of transmittable viruses, notably HIV.

BCT was externally monitored by the prothrombin time test in a series of regular, multicentre exercises to ensure reproducibility between batches (Poller 1970, Stevenson 1978). The shelf life of BCT was stated to be six weeks. The data in this chapter indicate that this was a conservative and safe choice.

The morphology of the reagent is depicted in figures 7.1 - 7.4. The low power electron micrograph (fig 7.1) shows two nuclei and many myelin figures. No subcellular organelles survive the extraction procedure, apart from nuclei. Although the background of the figure is filled with membranous structures these are likely to be vesicles of membrane formed from fragments broken from the myelin sheath membrane rather than subcellular formed elements. Changes to

myelin sheath membrane are evident in figures 7.2 - 7.4, where a degradation with time of storage at 4°C, with loss of order in the membrane organisation increasing with time, can be noted. The oldest preparation, BCT 1, stored for 102 months is remarkable in that some segments of the sheath membrane are still tightly associated.

The experiments in Chapter 7.3 demonstrate that an autoxidative process is responsible for the change in the lipid components of the stored reagent. With increasing time of storage the concentration of several phospholipid classes decreased, e.g. PC, PE, PS and PI. Free fatty acids and mda increased over the same period. Phospholipid-bound fatty acids altered too, showing loss of unsaturated components. Several highly significant correlations were seen between these changes and loss of procoagulant activity. It may be that a combination of potentially oxidative compounds in the reagent, combined with an inability to deal with radical production, leads to production of mda and free fatty acids. Mda is able to crosslink phospholipids and proteins, and will combine with amino acids. Free fatty acids may inhibit interaction of clotting proteins with thromboplastic surfaces which themselves will be altered by the loss of the zwitterionic phospholipids PC and PE, and the negatively charged classes PI and PS. The significance of these phospholipids in the clotting sequence is examined further in Chapter 8.

CHAPTER 8 EXPERIMENTS ON PARTIAL THROMBOPLASTIN

This chapter is concerned with attempts to elucidate the morphology, lipid composition, and performance in the activated partial thromboplastin time test, (APTT), of a number of widely used commercially available methods and the Manchester APTT. The aim is to develop a reagent, made from pure lipids, which may substitute for these and, moreover, which may have a role as a possible International Reference Preparation for the APTT.

8.1 EXAMINATION OF THE STRUCTURE OF SOME COMMONLY USED APTT REAGENTS

Aim - to determine whether APTT reagents show any formal structure by the use of electron microscopy.

Methods - Five APTT reagents, Actin, Actin FS, Activated Thrombofax, Automated APTT and Manchester APTT, were prepared for electron microscopy as described in Chapter 6.8. Four of these were produced by commercial manufacturers and were among the most popular APTT reagents in current use. The fifth was the Manchester APTT reagent, the most widely used in the UK, which has been evaluated in several international collaborative trials (Poller et al 1976, Poller et al 1980). Table 8.1 gives some features of the reagents.

TABLE 8.1 APTT REAGENTS EMPLOYED IN THE STUDY

Reagent (batch no)	Manufacturer	Source of Lipids
Actin (APAC-415B, APAC-429B	Dade	rabbit brain
Actin FS (SA-54B, SA-66C)	Dade	soya bean
Activated Thrombofax (3A443, 1B464)	Ortho	bovine brain
Automated APTT (3806062, 5328113)	General Diagnostics	rabbit brain
Manchester APTT (303)	UK Reference Lab	human brain

Ultra-thin sections of 50-60 mm thickness were prepared, with the exception of the Automated APTT reagent, which contained silica

particles as activator in the test system. Sections of this material were cut at a thickness of 100 nm.

Results - the morphology of the reagents is depicted in figures 8.1 to 8.5. Actin (fig 8.1) contains small fragments of membranous material, vesicular or tubular in structure, but poorly organised and widely dispersed. Sheets of electron dense and medium electron dense material are interspersed with the membranous components. Actin FS (fig 8.2) shows a more even distribution of membranous vesicles with single, double or occasionally multilamellar walls. The liposomes are of a fairly restricted size range and no non-lipid material is evident. The question of sizing is evaluated in the next section. Activated Thrombofax (fig 8.3) contains a great deal of non-lipid material. Myelin sheaths are to be seen, comprising tightly associated membranes, surrounding nerve axons often containing microtubules. The surrounding material is membranous in nature, containing vesicles ranging from small unilamellar to large multilamellar structures. Automated APTT looks markedly different from the other reagents. Its structure resembles a dense foam or honeycomb, comprised of electron dense wall material surrounding many fairly uniformly sized vesicles. Occasional large electron-lucent vacuoles are present, probably due to the loss of this reagents silica activator during preparation for microscopy. Automated APTT is shown in fig 8.4. The Manchester APTT is shown in fig 8.5. This preparation is characterised by the presence of discrete liposomes of varying complexity and size, from small unilamellar forms to large multilamellar. There is no evidence of non-lipid contaminants.

Fig. 8.1 (opposite) Electron micrograph of Actin at a magnification of 32 000. Scale bar is 500 nm.

Fig. 8.2 (p231) Electron micrograph of Actin FS at a magnification of 22 000. Scale bar is 500 nm.

Fig. 8.3 (p232) Electron micrograph of Activated Thrombofax at a magnification of 32 000. Scale bar is 500 nm.

Fig. 8.4 (p233) Electron micrograph of Automated APTT at a magnification of 50 000. Scale bar is 500 nm.

Fig. 8.5 (p234) Electron micrograph of Manchester APTT at a magnification of 20 000. Scale bar is 500 nm.

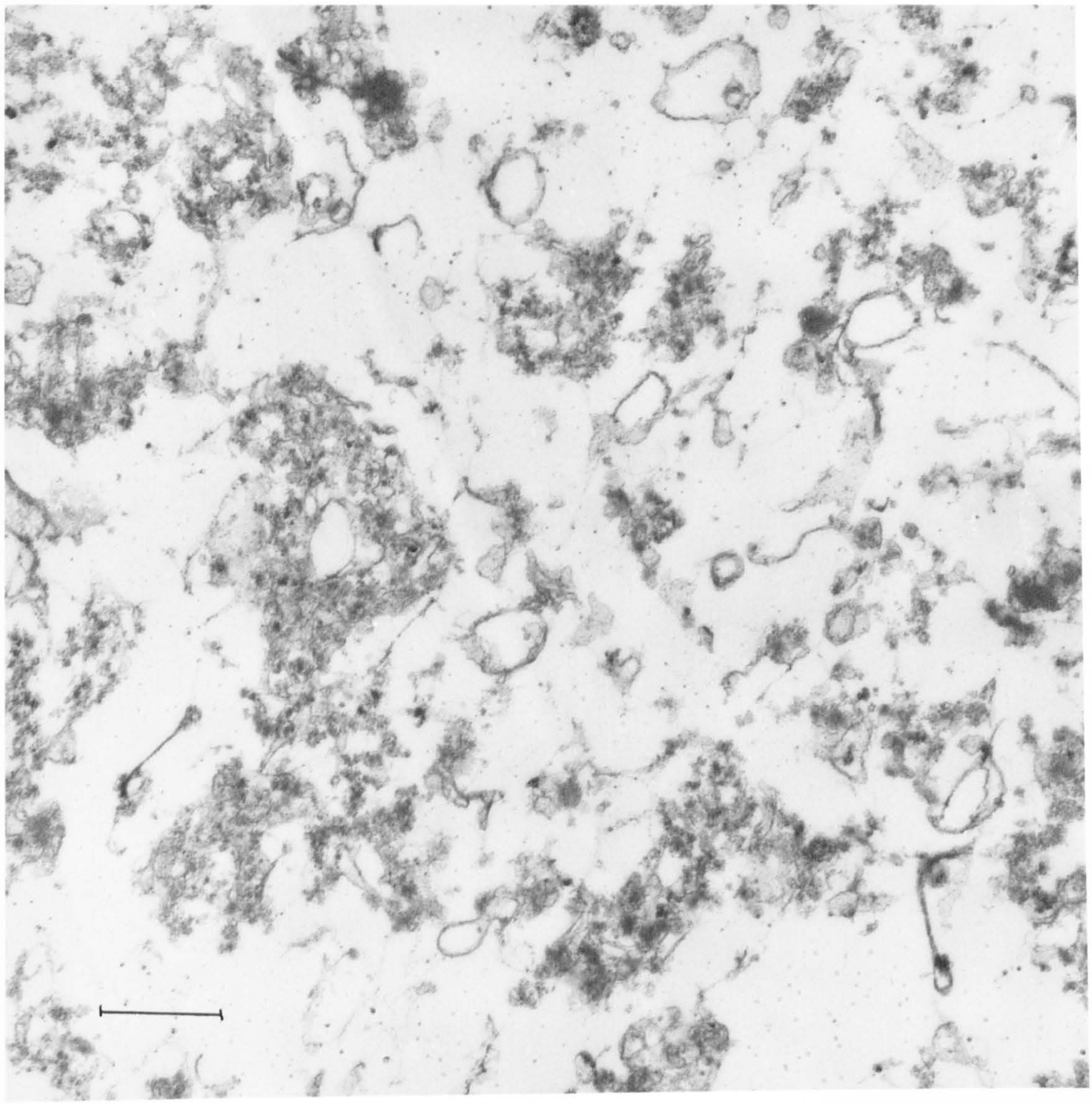


Fig 8.1

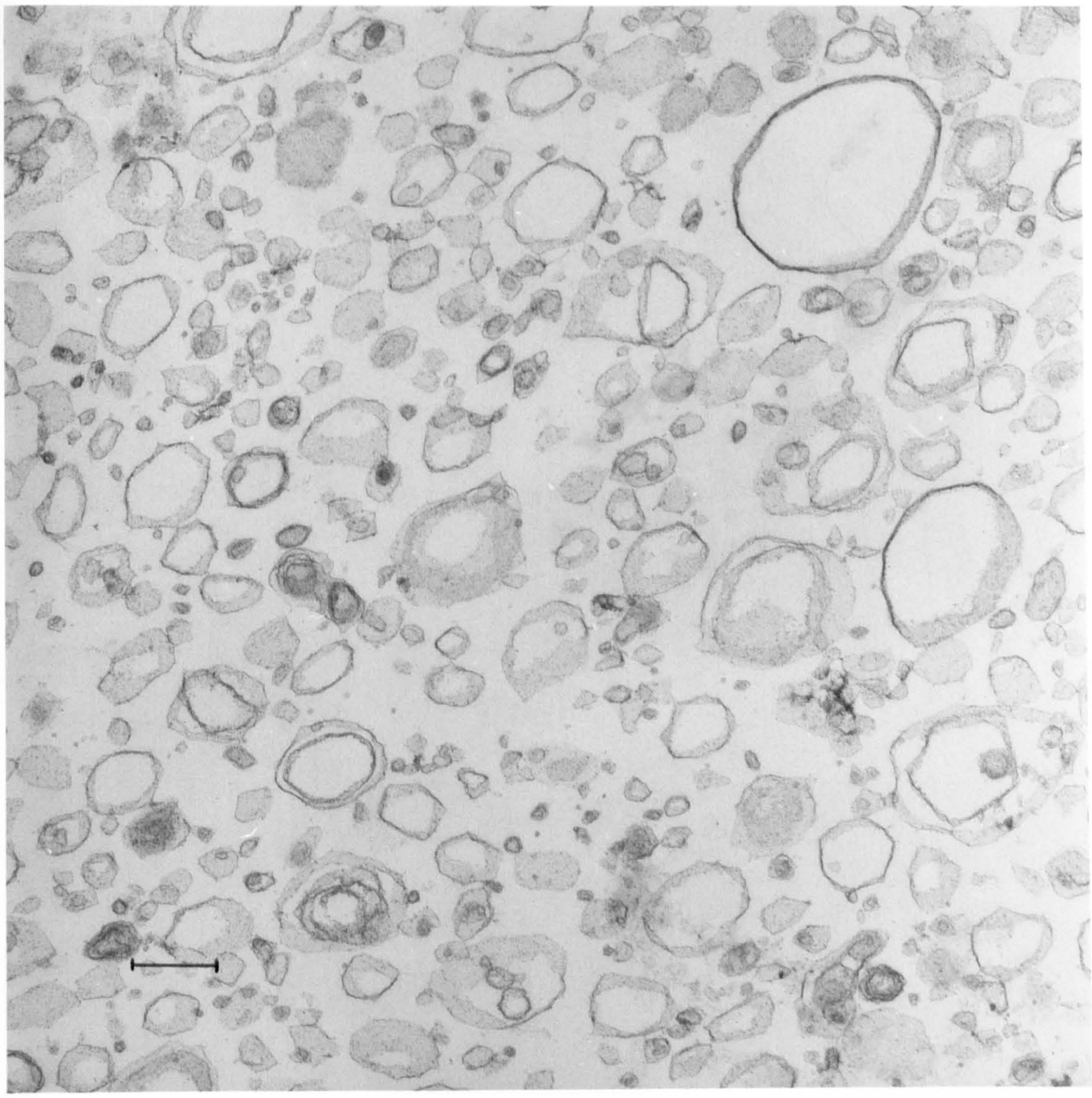


FIG 8.2

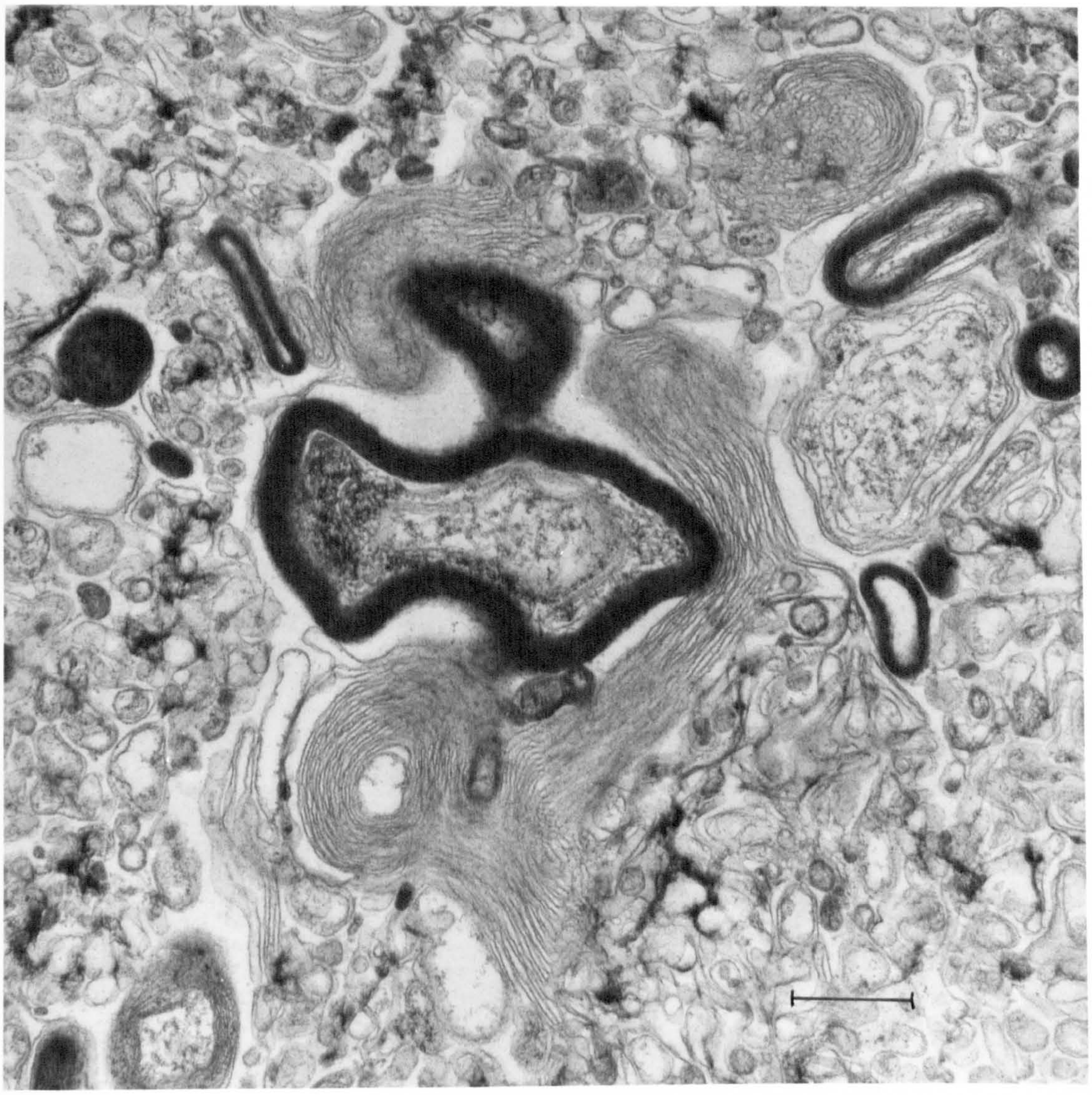


Fig 8.3

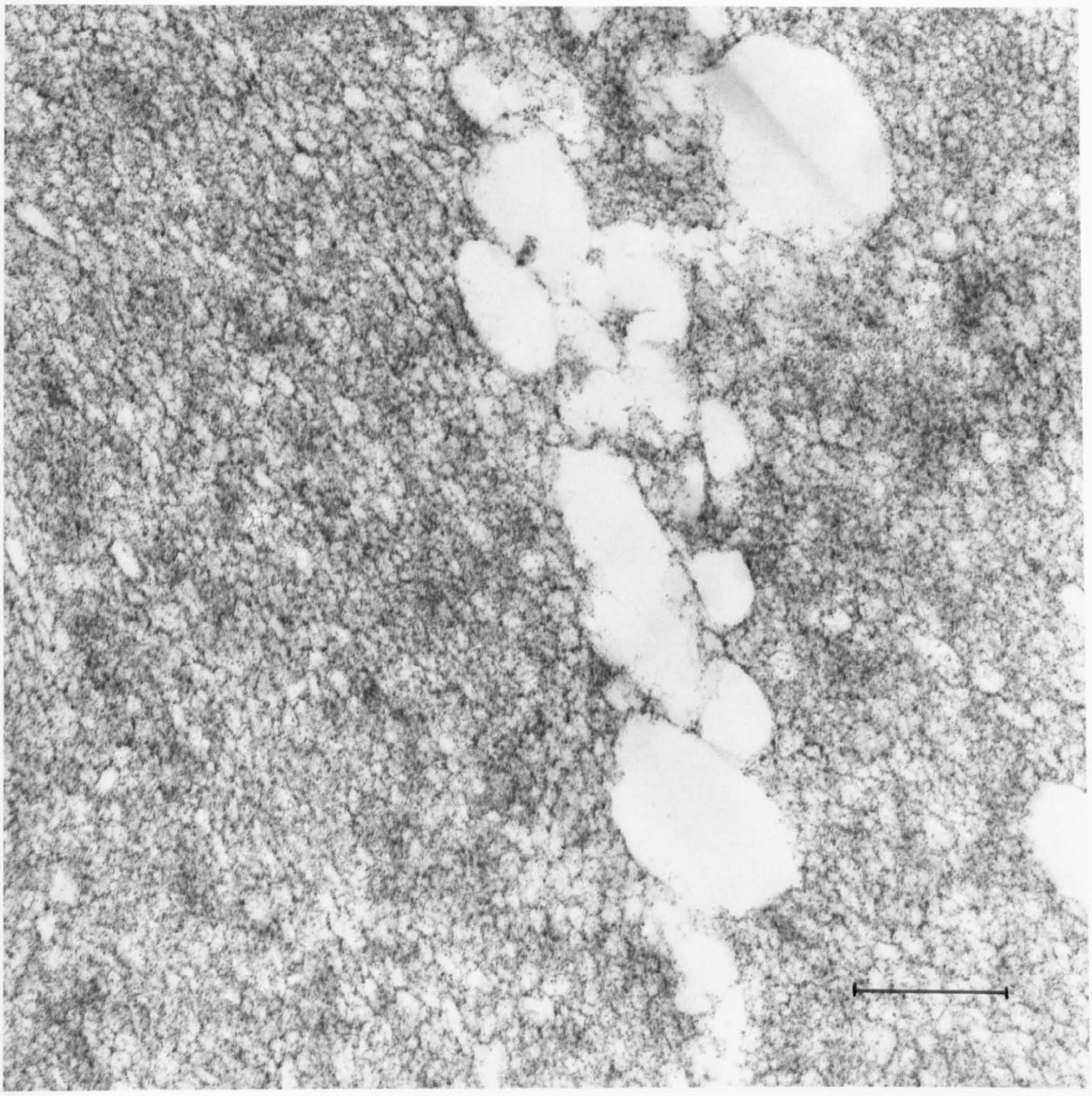


FIG 8.4

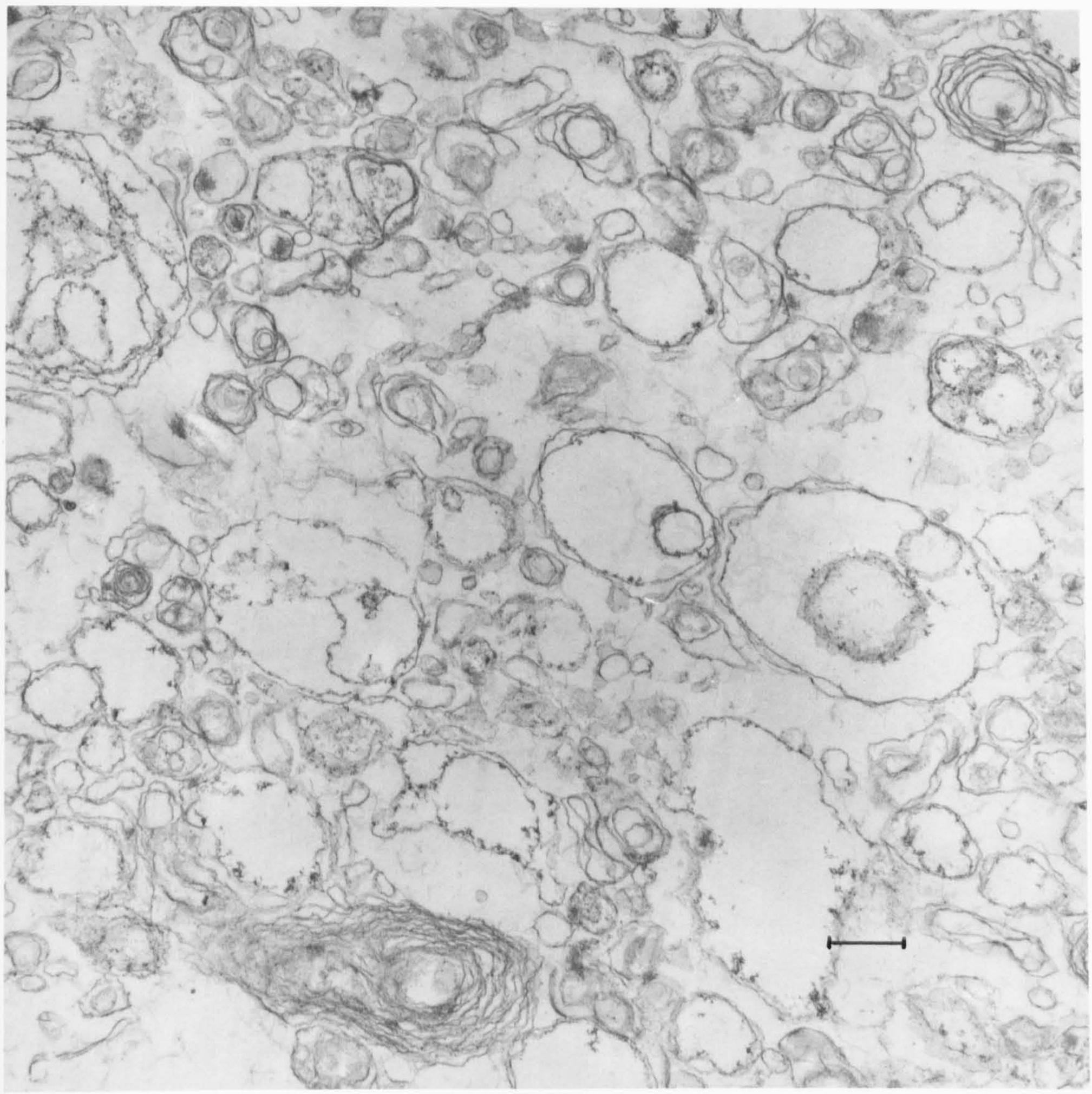


Fig 8.5

Discussion - the electron microscope examination shows that APTT reagents possess a discrete structure. The liposomes that comprise these preparations come in many sizes and forms, ranging from small unilamellar vesicles to large multilamellar vesicles built up of whorls of membranous material. Actin FS and Manchester APTT appear to have the most ordered structures. From the morphology, neither contains any non-lipid contaminants and their constituent liposomes are discrete and highly ordered. Actin FS appears to be more homogeneous in its size distribution (see Ch 8.2) than any of the other preparations. Manchester APTT is prepared from human brain lipids while Actin FS is made from soya bean. None of the other three reagents are as highly ordered as Manchester APTT and Actin FS. Actin and Automated APTT are both manufactured from rabbit brain lipids. They do not resemble each other however, with Actin shown to be widely dispersed vesicles of small size interspersed with membranous sheets while Automated APTT appears as a dense foam with electron lucent vacuoles. This may be due to the loss of the silica particles employed as activator in this reagent, which may fall out during processing.

The remaining preparation, Activated Thrombofax, was prepared from bovine brain and contains large fragments of nerve cells, nuclei and myelin sheaths. This indicates that insufficient care was taken to remove these contaminants, which may adversely affect the clotting time test in which the preparation is used, by contributing non-lipid components. Recent concern over bovine spongiform encephalopathy may also influence the choice of this

particular reagent (Scott 1988). A common feature of all of these preparations is that they are comprised of liposomal type structures which are highly suited to the provision of a surface for the reaction of the blood clotting proteins.

Conclusions - all the reagents examined were in the form of liposome suspensions. Two of the reagents, Actin FS and Manchester APTT show a more homogeneous appearance than the others, while Activated Thrombofax appears to be a crude extract containing a good deal of cell debris.

8.2 TO DETERMINE THE SIZE OF THE LIPOSOMES IN SOME COMMONLY USED APTT REAGENTS

Aim - to estimate the size range of some APTT reagent liposomes.

Methods - the APTT reagents described in Chapter 8.1 were examined using a Zetasizer 2 instrument in the case of Automated APTT and Manchester APTT and an Autosizer 2 instrument with Actin, Actin FS and Activated Thrombofax. These machines are described in Chapter 6.9 and 6.10.

Results - There is a wide range of particle size over all the reagents examined and each reagent also shows a wide size distribution. Table 8.2 shows particle size distribution in the reagents.

TABLE 8.2 PARTICLE SIZE DISTRIBUTION IN APTT REAGENTS

Range	Particle size distribution (nm) mean	range
Actin	166	65 - 800
Actin FS	269	120 - 640
Activated Thrombofax	132	50 - 800
Automated APTT	460	50 - 1250
Manchester APTT	938	65 - 2500

A typical Zetasizer output and Autosizer output are shown in figs 6.10 and 6.11. As may be seen in table 8.2 Actin FS has the smallest size range of any of the materials tested while the

Manchester APTT shows the largest. This reagent also contained the largest mean particle size. The smallest mean particle size was found in Activated Thrombofax.

Discussion - The method of extraction to obtain the phospholipid is only known for the Manchester APTT, produced in the UK Reference Laboratory. These details are not available for the four commercial reagents. Manchester APTT was manufactured by extracting acetone dried human brain powder with diethyl ether. The ethereal solution was subjected to rotary vacuum evaporation, depositing the lipids as a thin film on the walls of a round bottomed flask. Owren's buffer was added and the flask shaken by hand until the walls of the flask were freed of lipid. The electron micrograph (fig 8.5) and the data for size distribution in table 8.2 show that a variety of forms and sizes results from this procedure.

The finding that the other four reagents have narrower size ranges may be due to the way in which they were produced, e.g., it is unlikely that hand shaking of liposomes occurs in an industrial scale technique (Fildes 1981). More vigorous shaking, for example, by mechanical means, would result in smaller sized liposomes (Sjoka and Papahadjopoulos 1980). Alternatively, ultrasound probes or baths may be used to produce small sized particles and ultimately, narrower size ranges. Extrusion through polycarbonate filters or columns of Sepharose will also reduce the size range of liposome populations. Olson et al (1979) discuss the use of these methods. Sonication is difficult to standardize (Saunders et al 1962,

Papahadjopoulos et al 1967) so this group preferred a method employing extrusion through polycarbonate membranes.

The Malvern Instruments machines employed in the present study (see Chapter 6) proved useful for the size distribution estimation.

8.3 TO DETERMINE THE ELECTROPHORETIC MOBILITY OF APTT REAGENT LIPOSOMES

Aim - to obtain values for the electrophoretic mobilities of the liposomes of the five APTT reagents described in 8.1.

Methods - the Zetasizer 2 instrument was employed (see Chapter 6.10).

Results - all the reagents show a negative charge, Automated APTT being strongest, while Manchester APTT was weakest and similar to Actin FS.

Table 8.3 shows the electrophoretic mobilities of the reagent liposomes. Fig 6.9 shows a typical output from the Zetasizer 2 instrument.

TABLE 8.3 ELECTROPHORETIC MOBILITY OF REAGENT LIPOSOMES (microns/sec/volt/cm)

Actin	-3.65
Actin FS	-2.70
Activated Thrombofax	-3.24
Automated APTT	-4.68
Manchester APTT	-2.14

Discussion - the reagents all show a negative charge. Bangham (1961) prepared liposomes of egg lecithin containing graded concentrations of dicetyl phosphate. The electrophoretic mobility of these preparations was obtained by the use of the microelectrophoresis apparatus described by Bangham et al (1958). A Russels Viper Venom activated clotting time was then performed with

each of the liposome preparations in turn providing the phospholipid. Bangham found that as the negative charge on the lipid suspension increased so did its ability to accelerate the clotting time. Papahadjopoulos et al (1962) prepared a series of mixed phospholipid emulsions from beef brain and egg yolk. They used the microelectrophoresis apparatus described by Bangham to measure the electrophoretic mobility correlated with activity in a clotting test. They found that each phospholipid mixture had an optimal negative charge at which the particles exhibit their maximum activity. Variation of this charge resulted in diminished activity.

The microelectrophoresis method employed by these early workers involved directly observing individual particles using a microscope and timing their progress over a fixed distance. Considerable time and patience was required to accumulate a statistically satisfactory sample. The technique also required that particle concentration be reduced to allow an individual particle to be observed unequivocally for the duration of the measurement.

Use of the Zetasizer instrument provided a comprehensive assessment of the mobility spectrum of the entire sample without dilution and laborious microscopical monitoring of particle movement.

8.4 TO DETERMINE THE LIPID COMPOSITION OF FIVE COMMONLY USED APTT REAGENTS

Aim - to determine the phospholipid class composition, fatty acid composition and neutral lipid class distribution in the five APTT reagents described in Chapter 8.1.

Methods - the method used for determination of phospholipid and neutral lipid class distribution was thin-layer chromatography (TLC), with quantitation by densitometry. Gas liquid chromatography (GLC) on capillary columns was employed for the fatty acid analyses. Phospholipid assay was by the method of Stewart (1980) and phosphorus estimation was by the method of Rouser et al (1966). These methods are described in detail in Chapters 6.1, 6.4, 6.3 and 6.2.

Results - table 8.4 shows the total phospholipid in each reagent per test. The Manchester APTT contains the smallest quantity and Actin FS the largest. Fig 8.6 shows the total concentration, per test, of phospholipids, fatty acids and neutral lipids. It is clear that the Manchester APTT contains the least amounts of the majority of those components while Actin FS contains the most. The distribution of the phospholipid classes is pictured in fig 8.7 and in table 8.5. PC was the major lipid class in the vegetable reagent, Actin FS. SPH was not detected in this reagent whle PG and PA were present in reasonable amount. The other four reagents, of animal origin, contained no detectable PG or PA and had PE as their major phospholipid component. Table 8.6 shows the

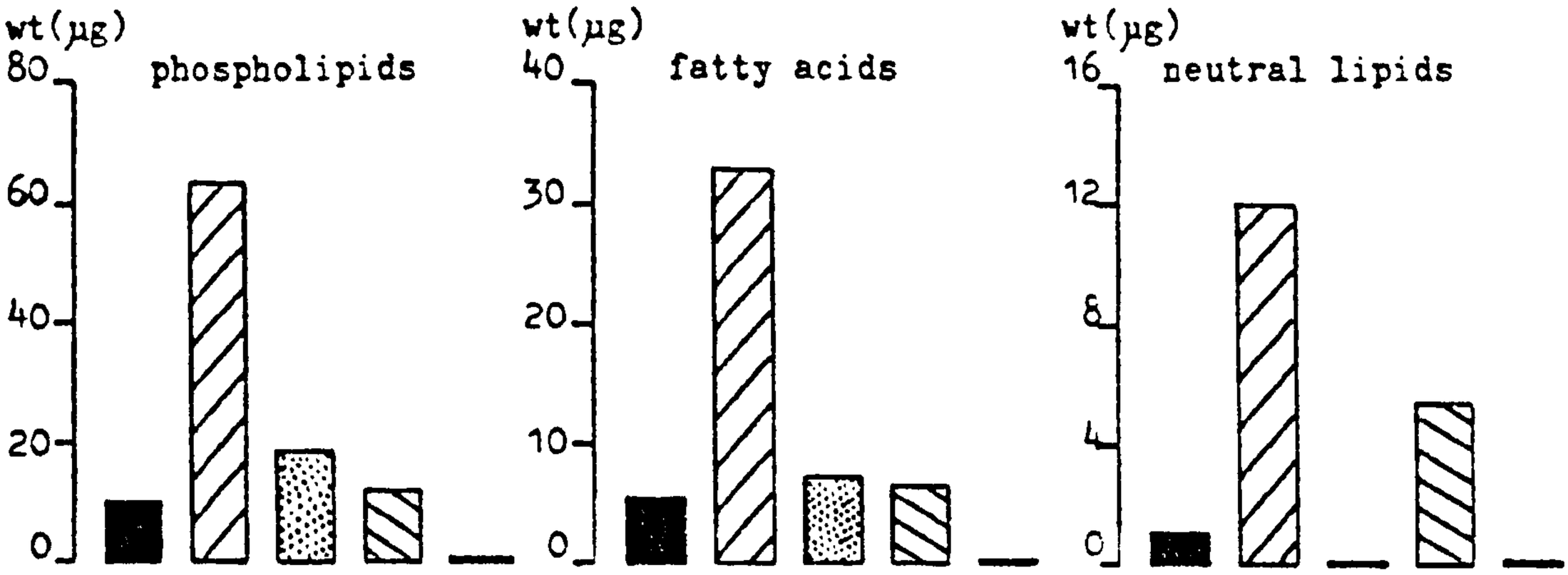
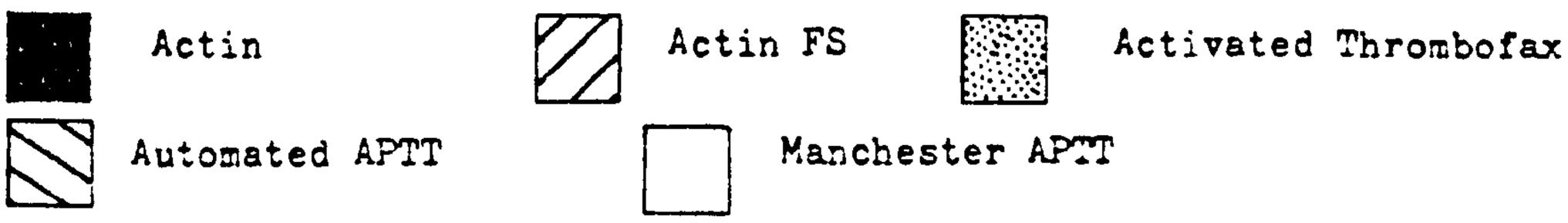


Fig. 8.6 Concentration, µg per test, of phospholipids, fatty acids and neutral lipids.



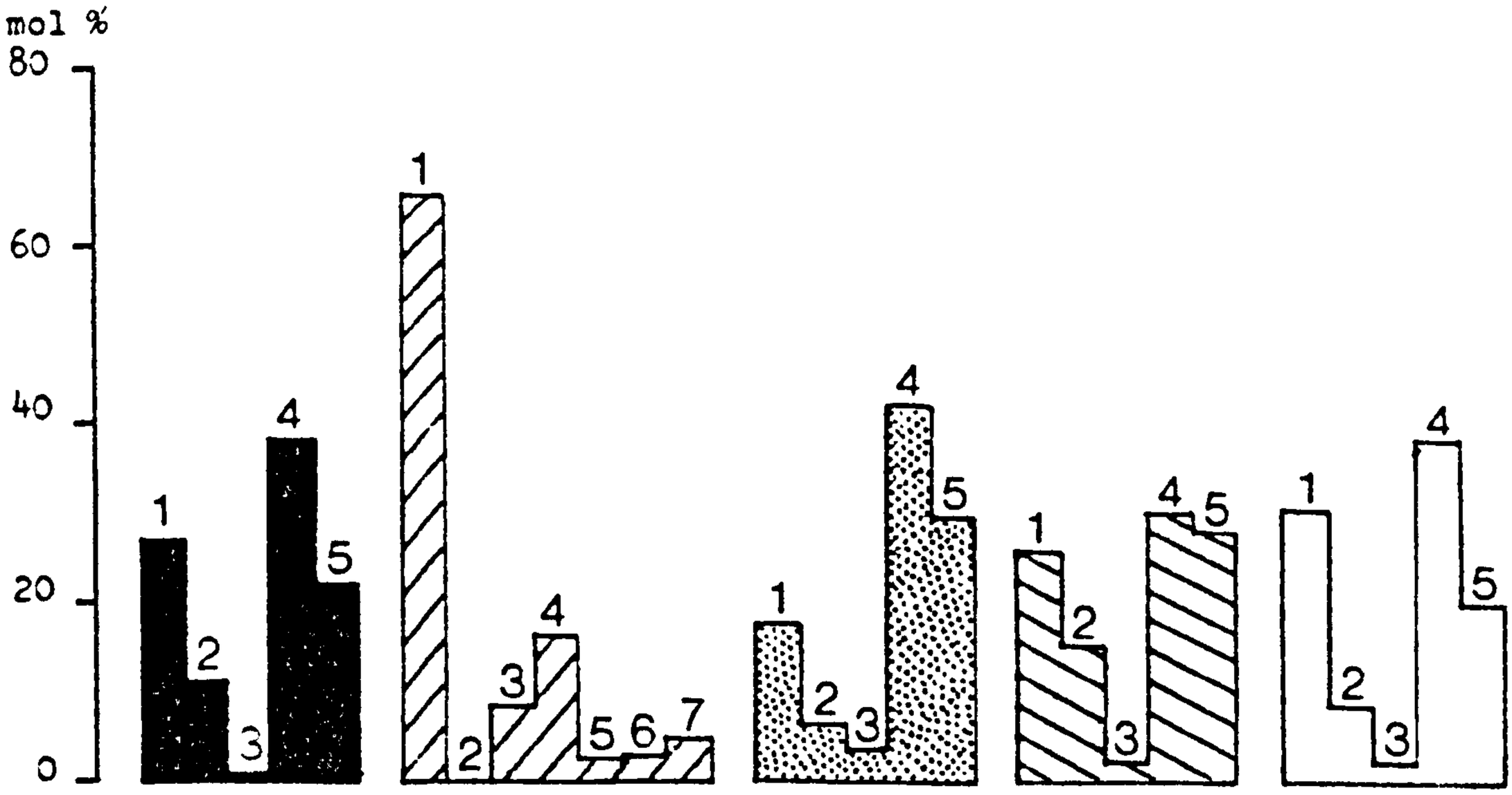


Fig..8.7 Phospholipid class distribution in APTT reagents 1 = PC 2 = SPH, 3 = PI, 4 = PE, 5 = PS 6 = PG, 7 = PA. Key as in fig 8.6

concentration of PS per test in the reagents. This indicates that the Manchester APTT reagent has the lowest concentration and Activated Thrombofax the highest. The concentration of the neutral lipids, per test, is shown in table 8.7. All reagents contained cholesterol esters and barely detectable quantities of free fatty acids and monoglycerides. Actin FS, the soya bean material, showed the presence of monogalactosyl diglyceride and digalactosyl diglyceride and small amounts of triglyceride were found in Activated Thrombofax and Manchester APTT. The fatty acid composition of the reagents is the subject of table 8.8 and fig 8.8. The ratio of unsaturated to saturated fatty acids (USFAR) is given and its highest value is seen to be 2.54 for Actin FS. Linoleic acid (18:2) was the major component of this reagents unsaturated fatty acid group. The other reagents USFAR range from 0.779 for Actin to 1.442 for Activated Thrombofax. In these reagents, of animal origin, the major unsaturated fatty acids were oleic (18:1), arachidonic (20:4) and docosahexaenoic (22:6). The major saturated fatty acid in the animal extracts was stearic acid (18:0). By comparison Actin FS contained less than 6% of this acid.

Discussion - the five APTT reagents employed in this study were used at their manufacturers' stipulated concentration. In order to make comparisons easier, the results for total phospholipid (table 8.4), total phospholipds, fatty acids and neutral lipids (fig 8.6) are given as values "per test". The main difference demonstrated is between Actin FS, which is of vegetable origin, and the other four reagents, made from rabbit, bovine and human brains. There was 253 times more total phospholipid in Actin FS than in Manchester APTT

although this factor is reduced to 41 times when relative concentrations of PS are compared. The phospholipid class composition shown in fig 8.7 reveals a broadly similar pattern in the four reagents of animal origin, with PE the major component. APTT reagents were known as "cephalin" reagents for many years, not unreasonably given that mixtures of PE with PS and PI were also known as cephalin by earlier workers (Thudichum 1894, Renall 1913, Baumann 1913). PC is the major phospholipid in Actin FS. Soya beans are reported to contain relatively large amounts of this component (Galliard 1973) but the present very high level observed may be due to the addition of an extra quantity of PC as a stabilizer. This use of PC was noted by Mead and co-workers in their studies of pulmonary surfactant (Wu et al, Huang 1969) and autoxidation of pure liposomes of soya bean PC (Wu et al 1982). PG and PA are to be found at 2.46 and 4.4 mol% respectively in this preparation. The form of liposomes is known to depend to a large extent upon the relative proportions of the phospholipid classes they contain (Szoka and Papahajopoulos 1981, Chapman 1984). All the preparations examined contain sufficient quantities of PC and PE, as well as SPH in the animal brain extracts, to form liposomes. Several other considerations are worth noting, e.g., Zwaal (1978) described instability of liposomes containing over 30 mol% of PS. Also, the presence of negatively charged phospholipids is essential for the binding of the clotting proteins via calcium ions. In the four reagents of animal brain origin the major negatively charged component is PS, which represents only a small percentage of the Actin FS phospholipid. This small proportion was increased, however, by the presence of PG and PA which were absent from the

other reagents. Despite its very high total phospholipid content, Actin FS provides just 1.55 μg of PS per test. The Manchester APTT provides 0.05 μg of PS per test. In Chapter 8.3 the question of electrophoretic mobility was considered, and it was shown that negative charge was a feature of all these reagent liposomes.

TABLE 8.4 TOTAL PHOSPHOLIPID PER TEST (μg)

Actin	Actin FS	Activated Thrombofax	Automated APTT	Manchester APTT
9.88	63.31	18.6	12.28	0.25

TABLE 8.5 PHOSPHOLIPID CLASS DISTRIBUTION (mol %)

	Actin	Actin FS*	Activated Thrombofax	Automated APTT	Manchester APTT
PC	27.23	66.0	17.73	25.61	30.78
SPH	11.22	-	6.70	14.65	8.76
PI	0.86	8.05	3.54	1.79	1.82
PE	38.59	16.43	42.51	29.99	38.58
PS	22.07	2.45	29.52	27.97	20.06
PG	-	2.46	-	-	-
PA	-	4.40	-	-	-

* In the case of Actin FS, some 6.5% by weight of phospholipid was not positively characterised. The mol% values in the table were calculated from the identifiable components.

TABLE 8.6 CONCENTRATION OF PHOSPHATIDYL SERINE PER TEST (μg)

Actin	Actin FS	Activated Thrombofax	Automated APTT	Manchester APTT
2.17	1.55	5.49	3.43	0.05

TABLE 8.7 NEUTRAL LIPID PER TEST (μg)

	Actin	Actin FS	Activated Thrombofax	Automated APTT	Manchester APTT
Cholesterol	0.225	-	-	0.725	0.076
Cholesterol Esters	0.750	5.9	0.07	4.600	0.001
Free Fatty Acids	tr	tr	tr	tr	tr
Monoglycerides	tr	tr	tr	tr	tr
Monogalactosyl Diglyceride	-	2.4	-	-	-
Digalactosyl Diglyceride	-	3.7	-	-	-
Triglycerides	-	-	0.09	-	0.005

tr = trace observed on TLC plate

TABLE 8.8 FATTY ACID COMPOSITION (% of total fatty acids, by weight)

Fatty Acids	Actin	Actin FS	Activated Thrombofax	Automated APTT	Manchester APTT
Unsaturated					
14:1	0.37	-	-	-	-
16:1	1.83	0.11	0.99	0.61	0.64
18:1	19.64	9.03	26.97	25.39	30.19
18:2	1.74	57.11	0.30	0.68	0.72
18:3	4.09	3.73	0.66	1.05	1.25
20:1	1.51	0.34	1.60	1.46	3.15
20:2	1.18	0.50	0.50	0.83	0.13
20:3	0.14	0.02	0.93	1.13	1.33
20:4	7.64	0.43	11.56	13.52	8.43
22:1	0.95	-	0.52	0.47	0.12
22:2	0.47	0.44	0.17	0.04	0.42
22:6	4.24	0.04	14.85	9.92	9.09
24:1	-	0.01	-	0.18	-
Saturated					
10:0	-	-	-	0.02	-
12:0	1.21	0.07	0.50	0.33	0.18
14:0	2.43	0.12	0.58	0.30	0.76
16:0	24.42	20.95	7.77	15.83	13.43
18:0	26.00	5.68	30.95	27.01	28.93
20:0	1.69	0.37	0.63	0.72	1.07
22:0	0.24	0.67	0.52	0.49	0.16
24:0	0.13	0.38	-	0.02	-
26:0	0.08	-	-	-	-
Ratio of Unsaturated: Saturated Fatty Acids	0.78	2.54	1.44	1.24	1.25

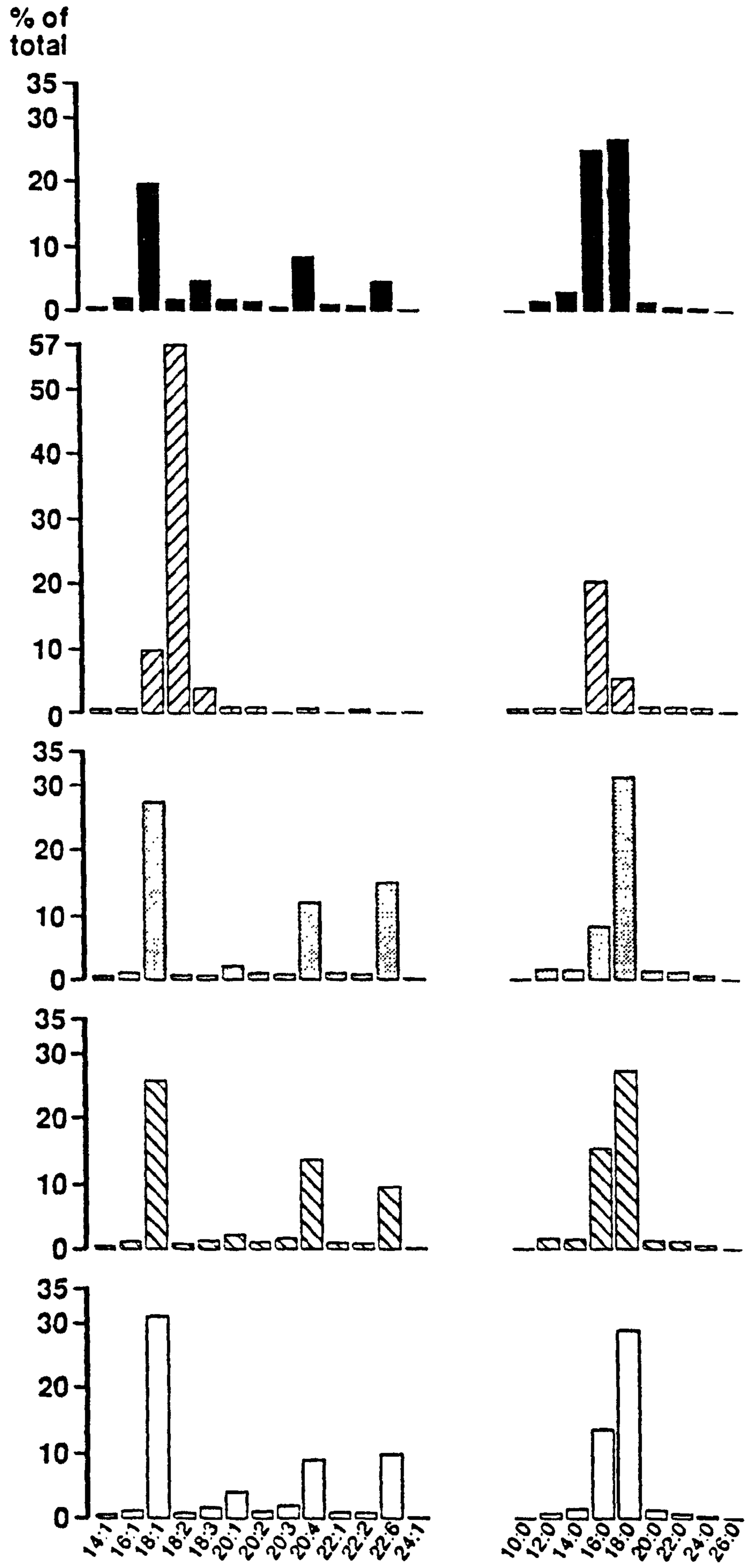


Fig 8.8 Fatty acid distribution in APTT reagents.

Key as in Figure 8.6

8.5 TO OBSERVE THE PERFORMANCE OF FIVE APTT REAGENTS IN TESTING THE APTT OF A VARIETY OF NORMAL AND ABNORMAL PLASMAS

Aim - to observe the performance of the five APTT reagents described in Chapter 8.1 in testing the APTT of plasmas from a variety of normal and abnormal sources.

Methods - APTT tests were performed in accordance with manufacturers' recommended techniques, using a manual method. Tests were carried out on plasmas obtained from 62 normal healthy donors and from patients with a range of clinically important congential and acquired coagulation abnormalities. There included 6 mild and 4 moderate to severe haemophiliacs, with factor VIII levels ranging from < 1% to 26%, 2 von Willebrand's disease patients, 3 factor XI deficiencies, 5 factor VIII inhibitors, 7 lupus-like inhibitors and 22 heparinised patients. In addition, the effect of heparin in vitro was studied over a range from 0.1 to 0.35 units cm^{-3}, by addition of standard heparin (3rd.International Standard prepared at the National Institute for Biological Standards and Control). The blood was collected and platelet poor plasma obtained as described in Chapter 6.19.

Normal ranges for each reagent were derived, also as described in Chapter 6.21. Clotting time ratios were obtained by method A or method B, as follows:- A was the APTT of abnormal plasma/upper limit of manufacturers' normal range and B was the APTT of abnormal plasma/upper limit of normal range which had been transformed to correct non-Gaussian distribution. The APTT ratios of the

individual plasmas with each of the five APTT reagents were ranked, a value of 5 being given to the highest and 1 to the lowest. Within each coagulation defect the ranks were summed for each reagent and the sums converted to a % scale (see Chapter 6.21 for further explanation).

Results - table 8.9 shows the results of the testing of 62 normal plasmas. The distrubtion of these normal values showed some skewness and kurtosis and therefore required transformation (Box and Cox 1964, Wetherill 1981). Statistical methods are described in Chapter 6.21. Corrected normal ranges were calculated from the transformed data and are shown with the manufacturers stated values in table 8.10. Also included is a normal range based on the data from this study, before transformation, for comparison. It may be seen that for the Manchester APTT these three ranges are very similar. Actin and Actin FS gave a narrow normal range and short times. Manchester APTT gave much longer times and a wider range. Limits of the normal clotting times with the different reagents varied from extremes of approximately 22 sec, the lower limit with Activated Thrombofax, to 48 sec, the upper limit with Manchester APTT.

TABLE 8.9 APTT OF NORMAL PLASMA

Actin		Actin FS		Activated Thrombofax		Automated APTT		Manchester APTT	
30	30.5	37	37	26	25.5	35	34.5	45	45
27	26	31	30	23.5	23	28.5	29.5	37.5	37
28	28	34.5	35.5	24	24.5	29	29.5	39	39
32	31.5	39	39	26.5	26	36	36.5	47	46
29	29.5	35	34.5	26.5	27	31	32.5	43	43
31	31	37.5	37	26.5	26.5	34.5	35	42.5	43.5
28	27.5	33	32.5	24	24.5	30	30	40	40.5
30	30	37	37	27	26	34	35	43	43.5
30	30	37	36	24	24	35	35	46	46.5
30	30	35	35	27	26.5	34	34.5	41	41.5
29.5	30	33	33	25	24	34	34.5	41	42
28	28	33	33	24.5	25.5	31.5	31.5	42	42.5
31.5	31	36.5	36.5	27	27	34.5	35.5	44	44
29	29.5	34	34	26	26	32.5	32.5	42.5	43
31	31	37	37	26	26	37	37	43	43.5
30	29.5	34	33	25.5	25	33	33.5	39	39
24.5	25.5	28	29	22.5	23	28	28	37	37.5
32	33	36.5	37	28.5	28.5	40	39.5	44	43
28	28.5	32	31	24.5	25	30	31.5	39	39
32	31	35	36	28.5	27.5	37.5	37	45	45.5
27.5	26.5	32	32	23.5	23.5	31	32	37	37
28.5	29	36	35	25.5	25.5	33	33	40	41
32	33	38	37	28	27	38	39	46.5	47.5
28.5	28	35.5	35.5	26	26	33	34	42.5	43
28	29	33.5	33.5	26.5	26	35	34.5	44	45
27.5	28	35	35	24.5	24.5	34	33	37.5	38
32.5	33	39.5	38.5	27.5	28	43	43.5	46	46.5
32	31	36	35.5	27	27	38	37	46.5	47
29	29.5	34.5	34	26.5	25.5	34	34	43	44
28.5	29	36.5	36	25.5	24.5	35.5	35	44	45
30.5	30	34	35	26.5	25.5	34	33	40.5	41
32	32.5	39.5	39.5	27.5	27	36	36	45	45.5
27	27.5	33.5	33	23	22.5	30	30	38	38
26	25.5	31	31	24	24	29	30	37	36.5
30	30	37	36	26	26.5	35.5	36	41.5	42
28.5	29	34.5	34	24	24	34	33	46	46.5
26	26	33	32	23.5	23.5	31	31	39	39.5
32	32	38	37.5	27	26.5	38	37	46	46
28.5	28.5	32	33	24	25	31.5	32	39.5	39
29	28	30	30.5	24	24	34	33.5	37	38
27	27	32	32	23.5	23	29	30	37.5	38
28	28	33	33	25.5	25	33	32	43.5	44.5
26.5	27	31.5	32	24	24.5	30	30	42	42
27	27	33	33	22	21.5	27.5	27.5	42	42.5
26	26	30	29	23	23.5	25	24	36.5	37.5
29.5	29.5	33	33	22.5	23	27.5	28	41.5	42.5

TABLE 8.9 APTT OF NORMAL PLASMA (continued)

Actin		Actin FS		Activated Thrombofax		Automated APTT		Manchester APTT	
26.5	27.5	31	31	22.5	22	27	27	41	41
30	29.5	35	36	26.5	27	35.5	35	42	43
32	31	39	40	26	27	37.5	38.5	45	46
28	28	36	37	25	25	32	32	42.5	43
29	29.5	37	37.5	25.5	26	35	36	46.5	46.5
33	33	38.5	39.5	28	28	37	38	44	45
32.5	32	37	36.5	26	26.5	37	36.5	43.5	44.5
33	33	40	40	28.5	29	40	40	46	46.5
31	31	39	38.5	26.5	27	36	36	44.5	45.5
27.5	26.5	33.5	33.5	24	23	31	31	39	39
28	27.5	35	34	24.5	23.5	30.5	31.5	39.5	40
31	30	35	35	26	25	37	36	43.5	44.5
30.5	30.5	35.5	35.5	27	27	37	36	44	45
31	31	36	37	27	26	39	38	43	43.5
26	25	30	31	23.5	24	28	27.5	36	36.5
31	32	36.5	37	23.5	23	30.5	29.5	49.5	50.5

TABLE 8.10 NORMAL RANGES OF THE APTT METHODS (sec)

Reagent	Manufacturer's range	Range of normal plasmas tested (n = 62)	Corrected range
Actin	25.50 - 34.70	25.26 - 33.41	25.20 - 32.91
Actin FS	29.20 - 37.10	29.29 - 40.04	29.33 - 39.81
Activated Thrombofax*	24.00 - 30.41	22.10 - 28.56	22.18 - 28.29
Automated APTT	25.00 - 43.00	25.61 - 41.07	26.37 - 39.39
Manchester APTT	36.00 - 48.00	36.00 - 48.62	35.59 - 47.89

*normal range for manual technique not given. The range shown is the average of those given for automated methods.

Fig 8.9 shows the distribution of the normal values obtained when using Actin FS. The manufacturers given normal range is shown by the solid arrows and excludes several data which are normal and therefore should be included. The corrected range, following the transformation described in Chapter 6.21 is shown by the dotted arrows.

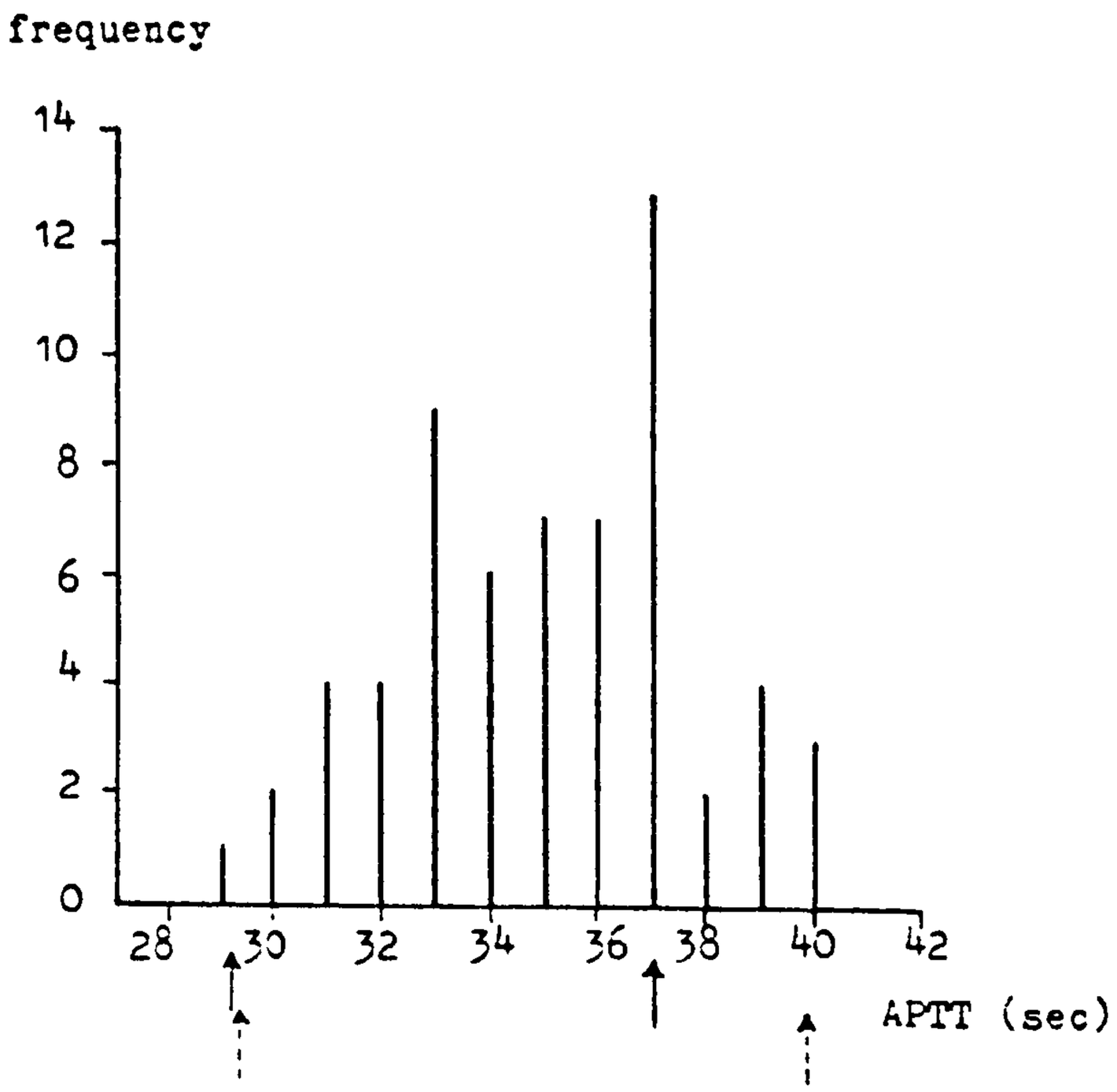


Fig 8.9 Frequency distribution. APTT of normal plasmas with Actin FS. Solid arrows show the manufacturers given range while the dotted arrows show the transformed range.

Tables 8.11 to 8.17 show the performance of each of the APTT reagents with the different coagulation abnormalities under test.

The bar charts (fig 8.10) indicate the ranking of the reagents with each coagulation defect. Results based on manufacturers' normal values are shown alongside those derived by use of a corrected normal range. Table 8.18 shows the number of abnormalities detected by the various APTT methods according to the upper limit of the manufacturers' normal range (A) and that calculated in the present study (B).

The APTT methods differed in their ability to detect the range of coagulation defects included. The correction of the normal range improved the success rate of Actin, Activated Thrombofax and Automated APTT in detecting mild coagulation abnormalities. In mild haemophilia all the reagents detected the abnormality in all samples, but the ease of detection indicated by the ranking suggests that Automated APTT and Activated Thrombofax were the least sensitive and Actin FS the most. In severe haemophilia Actin FS and Manchester APTT performed well whereas Automated APTT and Activated Thrombofax were again the least sensitive. A similar pattern was evident in von Willebrand plasmas when the results were based on the manufacturers' normal ranges. Automated APTT failed to detect the abnormality in both plasmas and Activated Thrombofax in one. Using the corrected values the performance of these two reagents was improved.

Fig. 8.10 (opposite) Ranking of reagents. Column A used ratios based on manufacturers given ranges while Column B used corrected ranges derived as described in the text. Key as in fig. 8.6

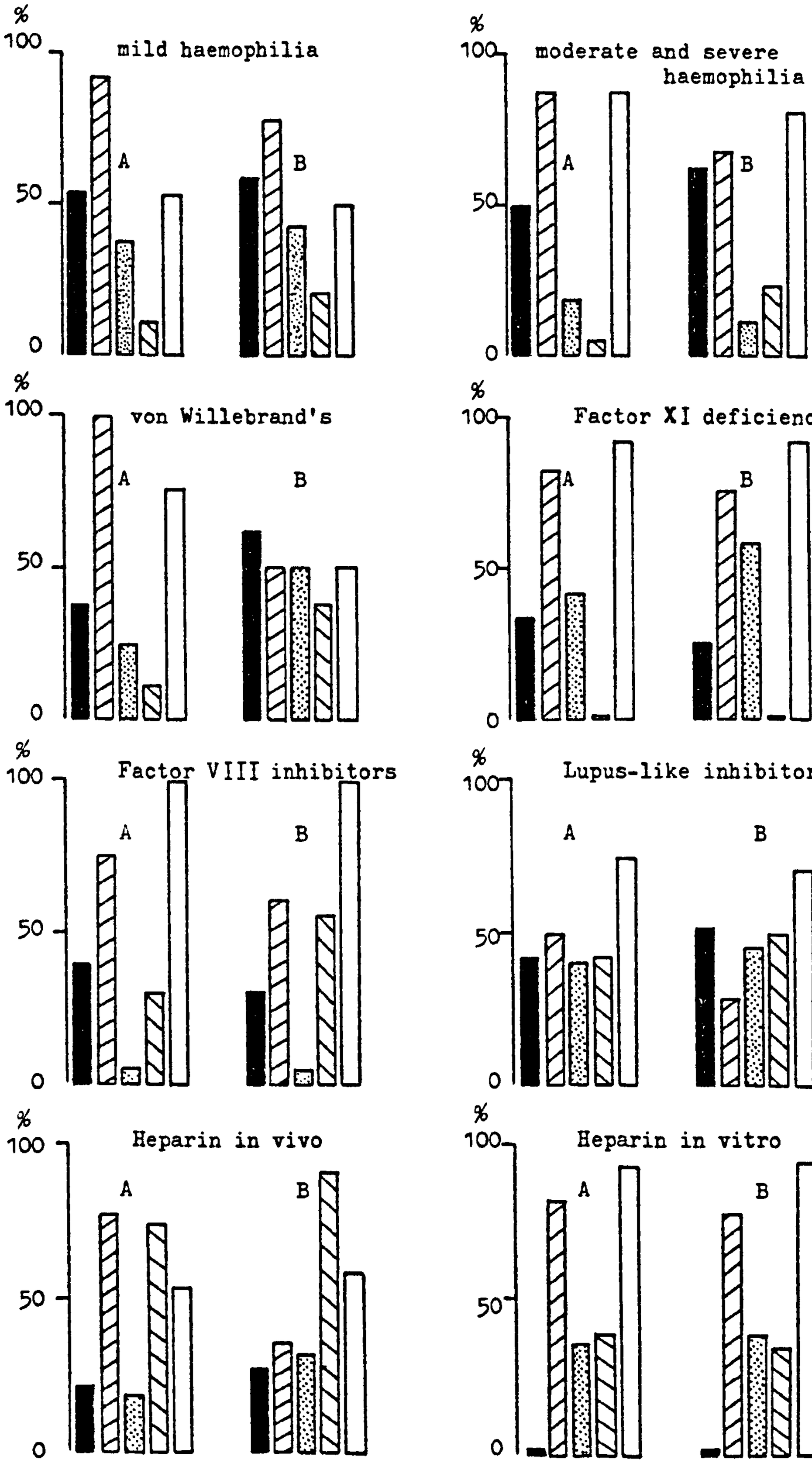

%
100
50
0
mild haemophilia
A
B
moderate and severe haemophilia
von Willebrand's
Factor XI deficiency
Factor VIII inhibitors
Lupus-like inhibitors
Heparin in vivo
Heparin in vitro

Fig 8.10

TABLE 8.11 APTT OF FVIII DEFICIENT PLASMA

	Actin		Actin FS		Activated Thrombofax		Automated APTT		Manchester APTT	
FVIII level %										
26	42.5	44	56.5	57	37	37	49	51	54	55
13	37.5	38	51	50	33	32.5	45.5	45.5	61.5	62.5
4	59.5	58.5	79	80	42.5	43	56.5	58	90.5	93
<1	79.5	78	90	93	47.5	49	94	95	192.5	191.5
12.2	44	44.5	50	50	36	35.5	46	46	68	68
10.5	46	46	60	59.5	37.5	38	53	54.5	65.5	66
9	45	44	57	57	35.5	36	49.5	48	62	62
24	41	42	57.5	57.5	48	46.5	47	47	52	51
<1	85	84	99	103.5	56.5	59	81	79	190	191.5
2.8	79	81	116	118	51.5	51.5	73	71	117	112

TABLE 8.12 APTT OF VON WILLEBRANDS PLASMA

Actin		Actin FS		Activated Thrombofax		Automated APTT		Manchester APTT	
36.5	36.5	42.5	42	30	29.5	41.5	43	50	51
37	37.5	46.5	47	33	33.5	42	42.5	55	54.5

TABLE 8.13 APTT OF FXI DEFICIENT PLASMA

Actin		Actin FS		Activated Thrombofax		Automated APTT		Manchester APTT	
39	39	53.5	53	34	33.5	41.5	42	57.5	58.5
90	87	177	182	116	111	83.5	85	246	254
49.5	50	69	68.5	53.5	54	57	58	94	95

TABLE 8.14 APTT OF FVIII INHIBITOR PLASMAS

Actin		Actin FS		Activated Thrombofax		Automated APTT		Manchester APTT	
118	119	156.5	149.5	64	64	133	141	257	258
94	97.5	114.5	118	52	53	122	127	271	270
95	93	109	104.5	54	54.5	122.5	123	268.5	268
81.5	79	99	100.5	49.5	51	97	96	160	162.5
57	56.5	70.5	71	49.5	49	69.5	69.5	119.5	120

TABLE 8.15 APTT OF LUPUS-LIKE INHIBITOR PLASMAS

Actin		Actin FS		Activated Thrombofax		Automated APTT		Manchester APTT	
44.5	44.5	61.5	60	45.5	44.5	58	59.5	76	78
38	39.5	45	46	32	32	45.5	46	50	52
43.5	44.5	63	63.5	46	46.5	60.5	59	84	82.5
58.5	60	42	43	38	37	60	61	56	57
49	49	72	74	46	47.5	85	87	108	106
29	29	30.5	31	27	26.5	32	32.5	51.5	53
49.5	49.5	39	39	34.5	35	56	57	75.5	75

TABLE 8.16 APTT OF PLASMAS FROM HEPARINISED PATIENTS

Actin		Actin FS		Activated Thrombofax		Automated APTT		Manchester APTT	
37	36	60	58.5	35.5	35.5	49.5	50.5	79	79
51	50	81	81	42.5	42.5	80.5	82	81	80.5
52	52	64	66	47	48	82	80	76	78
43.5	45	61	61.5	40	38.5	60	65	64	66
90	95	160	165.5	48.5	46.5	97	101	134	132
93.5	95.5	96.5	97	59	59	136	141	90	89.5
116.5	115	144	133	72	74	204	211.5	133	135
35	34.5	47.5	46.5	32.5	32.5	54	54.5	58	58.5
29.5	29.5	38.5	39	27	27	43	44	48.5	49
30.5	31	39.5	40	28.5	29	40.5	41	65.5	66.5
49	49	58	59	38.5	39	72.5	74	72.5	73
31	31.5	37	37	27.5	27.5	41	40.5	46.5	46.5
35.5	35	42	42.5	30	29.5	44.5	44	51.5	51.5
32.5	32.5	43.5	42.5	29	29.5	47.5	48	53.5	54
33	33.5	36.5	37.5	27.5	28	44.5	45	52	51
41	41.5	44	43	36	35	53	53	68.5	70.5
36	35	38.5	39	30.5	31	46	46	46.5	46.5
33	32	35	35	27.5	27	44	45.5	45	45
32	31.5	33	33	28.5	28.5	43	44	39	39
31	31.5	38.5	39	28	28.5	45.5	44.5	49.5	49.5
60.5	61.5	68	68.5	55.5	55	125	126.5	116	117.5
35.5	37	45	46	31	31	51	51.5	58.5	58

TABLE 8.17 APTT OF PLASMA WITH HEPARIN ADDED

	Actin		Actin FS		Activated Thrombofax		Automated APTT		Manchester APTT	
heparin added										
0.1 u/ml	35.5	36	57.5	57	38	37	48	49	78	79
0.2 u/ml	42.5	43.5	87	86.5	51	51	65	67	110	111.5
0.3 u/ml	52.5	54	133	135	61	62.5	83	83.5	138	140
0.1 u/ml	34.5	35	50.5	52	34	34	50.5	49.5	76	74.5
0.15 u/ml	39	37.5	63.5	63.5	40.5	40	55	54	102	101.5
0.3 u/ml	48	47.5	90.5	95.5	50.5	50.5	77.5	80.5	158.5	156.5
0.35 u/ml	49.5	49.5	96	96.5	55	55.5	93.5	96	130	131

TABLE 8.18 SUCCESSFUL DETECTION OF COAGULATION DEFECTS BY THE APTT METHODS

	Number tested	Actin		Actin FS		Activated Thrombofax		Automated APTT		Manchester APTT	
		A	B	A	B	A	B	A	B	A	B
haemophilic mild	6	6	6	6	6	6	6	6	6	6	6
haemophilic moderate and severe	4	4	4	4	4	4	4	4	4	4	4
von Willebrand's	2	2	2	2	2	1	2	0	2	2	2
FXI deficiencies	3	3	3	3	3	3	3	3	3	3	3
FVIII inhibitors	5	5	5	5	5	5	5	5	5	5	5
lupus-like inhibitors	7	6	6	6	5	6	6	6	6	7	7
heparinised patients	22	14	15	18	14	13	17	20	22	18	18
heparin in vitro	7	7	7	7	7	7	7	7	7	7	7

With factor XI deficiencies, Automated APTT was the least sensitive. With factor VIII inhibitors, Activated Thrombofax was least sensitive and Manchester APTT the most. With lupus-like inhibitors Actin FS gave the poorest response and Manchester APTT the best. The other reagents failed with one plasma. The order of ranking with heparin in vivo differed from that in vitro. In heparinised patients Automated APTT gave the best ranking. Activated Thrombofax and Actin were the least sensitive to heparin both in vivo and in vitro.

Discussion - this study reveals the extent of the differences in coagulation response between five of the most widely used APTT reagents and supports previous observations on their varied sensitivities to a spectrum of coagulation disorders (Sibley et al 1973, Hoffmann and Meulendijk 1978, Hathaway et al 1979, Mannucci 1982, Thomson and Poller 1985). The APTT methods included differ in several respects e.g. source of phospholipid (animal or vegetable, type and concentration of activator, type of buffer, presence or absence of protective additives and in their activation times. Some or all of these features may contribute to the variation in performance observed. It appears that calculation of the normal range should not be based simply on a mean value $\pm$ 2 SD, but should be calculated with a correction for non-Gaussian distribution, as with the Manchester APTT. When this approach is used the major change observed was a lowering of the upper limit of normal in Actin, Automated APTT and Activated Thrombofax, with a concomitant increase in success rate in the detection of mild or borderline coagulation defects. The upper limit of normal was increased for

Actin FS, however, which should reduce the incidence of incorrectly classified normal plasmas at the upper limit of the range.

The study also confirms that APTT methods exhibit variable abilities in the detection of coagulation abnormalities. The main function of the APTT test is to screen for intrinsic coagulation disorders. A reagent which is reliable over a wide range of coagulation defects would therefore be preferable. Ranking of the reagents was based on their relative performance within the individual coagulation defects. The rankings for the various defects have not been summed because a poor performance with one or more defects could be masked by good performance with others and may not be a reliable guide to the value of the APTT method as an overall screening procedure.

There were some inconsistent results over the spectrum of clotting defects with some reagents. For example, Automated APTT obtained a good score with samples from heparinised patients but was relatively poor in the detection of FVIII and FXI defects. On the other hand Actin FS performed well with FVIIIC defects but relatively badly with lupus-like inhibitors. The Manchester APTT performed reasonably well with all defects and gave the highest ranking with FVIII inhibitors, lupus-like inhibitors, moderate and severe haemophilia and FXI deficiencies.

The response to in vivo and in vitro heparin was in most instances paradoxical. A high ranking with in vivo studies was not necessarily reflected in vivo, or vice versa. In vitro studies were

included because of their popularity in measuring heparin effect by the APTT (Poller et al 1980, Stevenson et al 1983, Brandt and Triplett 1981, Triplett et al 1978, Shapiro et al 1977). The present study indicates, however, that the performance of APTT reagents on plasma samples heparinised in vitro cannot be regarded as a reliable guide to the results from heparinised patients.

8.6 TO DETERMINE WHETHER ANY STATISTICALLY SIGNIFICANT RELATIONSHIPS EXIST BETWEEN PERFORMANCE IN THE APTT TEST AND THE STRUCTURAL AND CHEMICAL CHARACTER OF THE TEST REAGENTS

Aim - to test for correlation between the sensitivity of the reagents to a variety of coagulation defects and normal values reported in 8.5 and observations on size distribution, electrophoretic mobility and lipid composition described in Ch 8.1 to 8.4.

Methods - the method employed was Spearman's Rank Correlation test (Spearman 1904, Siegel 1956) which is described in Ch 6.21. Rank in clotting performance was compared with

1 size of particles

2 electrophoretic mobility

3 total phospholipids (µg/test)

4 mol % PS

5 PS conc (µg/test)

6 mol % PC

7 mol % PE

8 mol % PI

9 mol % SPH

10 ratio of mol % PS to mol % PI

11 ratio of negatively charged phospholipids to total phospholipids

12 total neutral lipids (µg/test)

13 total fatty acids (µg/test)

14 ratio unsaturated/saturated fatty acids

Results - significant correlations between clotting studies and particle size, electrophoretic mobility and lipid composition, as listed above, are shown in table 8.19. These correlations were significant at the 5% level. There was a highly significant negative correlation between the quantity of PS per test and the normal control times; the less PS the longer the normal APTT. In testing of the severe haemophiliac plasmas a negative correlation was seen between APTT prolongation and mol % of PS, PS concentration per test and the ratio of negatively charged to total phospholipids. A strong positive correlation was seen between APTT prolongation and mol % of PC. Plasmas with FVIII inhibitors also showed negative correlations between APTT prolongation and PS concentration per test and ratio of negatively charged to total phospholipids. A strong positive correlation existed between APTT prolongation and liposome size within this group, the only correlation seen with particle size.

Negative correlations existed between APTT prolongation and electrophoretic mobility in the FXI deficient plasmas and plasma heparinised in vitro. A negative correlation was also seen between APTT prolongation in the plasmas with lupus-like inhibitors and the total concentration of phospholipid per test. There were no significant correlations between clotting performance and the concentrations of PE, PI, SPH, ratio of PS to PI, total neutral lipids or total fatty acids. Further, it was not possible to correlate the ratio of unsaturated to saturated fatty acids in the APTT reagents with their clotting performance.

TABLE 8.19 SIGNIFICANT CORRELATIONS BETWEEN CLOTTING PERFORMANCE AND LIPOSOME SIZE, ELECTROPHORETIC MOBILITY AND LIPID COMPOSITION

	Size	Mobility	Total phospholipid	Mol % PS	PS per test	Mol % PC	Ratio negative charge to total
normal					NEG		
FVIII:C def (<1.4%)				NEG	NEG	POS	NEG
XI def		NEG					
FVIII inhibitor	POS				NEG		NEG
lupus-like inhibitor			NEG				
heparin in vitro		NEG					

Discussion - the number of significant correlations between lipid content and reagents' procoagulant activities confirms the view that the performance of an APTT method is to a large extent dependent upon its lipid compositon (Stevenson et al 1983, Stevenson and Poller 1982, Barrowcliffe and Gray 1981a, 1981b, Slater et al 1980).

For example, the finding of a significant correlation between the concentration of PS and APTT prolongation in severe haemophilia supports the observations of Wagenvoord et al (1983) and Yashioka et al (1983) that PS appears to have a specific association with FVIII:C.

Features that influence charge i.e. proportion and absolute concentration of PS and ratio of negatively charged phospholipid to total phospholipid, together with measurement of electrophoretic mobility dominate the table of correlations (Table 8.19). These correlations emphasize the importance of negative charge on the reagent liposomes, a finding described by Bangham (1961) and Papahadjopoulos et al (1962).

The APTT test is the most common screening test for the detection of lupus-like inhibitors. The concentration of total phospholipid in the test is well known to govern the sensitivity of the method to the inhibitor (Kelsey et al 1984, Mannucci et al 1979). This was confirmed in the present study by the observation that the five reagents evaluated contained markedly different

amounts of phospholipid. Actin FS had the highest concentration and performed least well, whereas Manchester APTT, with the lowest phospholipid concentration gave the highest ranking.

Only one group of plasmas, the FVIII inhibitor group, showed any correlation with liposome size. This was a strong positive correlation i.e. APTT reagents with larger liposomes gave greater prolongation in the APTT test. Table 8.12 shows the size distribution in the various reagents and it is evident that there is a very wide range of size in each reagent, with considerable overlap. It might have been expected, therefore, that the reagents would perform in a broadly similar way, but this was not the case as fig 8.10 shows, with the Manchester reagent's performance being much better than that of Actin FS, Automated APTT and Actin whilst Activated Thrombofax performed relatively badly.

The work described in sections 8.1 to 8.6 formed the basis for a paper entitled "The Reliability of Activated Partial Thromboplastin Time Methods and the Relationship to Lipid Composition and Ultrastructure" published in Thrombosis and Haemostasis, vol 55, pp 250 - 258 (1986), a reprint of which is appended.

8.7 PREPARATION OF A LIPOSOME WITH NEITHER PROCOAGULANT NOR ANTICOAGULANT ACTIVITY

Aim - the value of a liposome with neither procoagulant nor anticoagulant activity in the APTT test would be considerable. Such a preparation could be used as a vehicle, into which other phospholipids could be incorporated to test their activity in the APTT test.

Procedure - stock solutions of dipalmitoyl PC, PE and Chol were prepared, at a concentration of 10 g dm^{-3} in chloroform/methanol (95:5 v/v). Aliquots from each stock were mixed in the proportion 1:1:2 by volume (equivalent to a molar ratio of 17.55:17.94:64.52). Liposomes were prepared as described in Chapter 6.6 and three aliquots removed for testing of purity by two dimensional TLC, determination of the USFAR by GLC and for electron microscopic examination. These techniques are also described in Chapter 6.1, 6.4 and 6.8.

The liposome, in the range of concentrations from 100 mg dm^{-3} to 10^{-5} mg dm^{-3} of total lipid, was used to determine the APTT of a normal plasma and the clotting activity expressed as % of batch 117 (see Ch 6.11).

Results - purity testing by TLC revealed no evidence of degradation of the liposome components, i.e., there were three spots on the plate, with no evidence of free fatty acids or

lysophospholipids. Table 8.20 shows the results from the TLC examination.

TABLE 8.20 LIPIDS EXTRACTED FROM 10 ml OF TEST LIPOSOME (100 mg dm^{-3} concentration)

dipalmitoyl PC	246 ug
PE	256 ug
Chol	485 ug

A number of conclusions may be drawn from this data. Firstly, there was no degradation of the lipids due to the manipulation required to prepare the liposome. Secondly, recovery of the individual components was good since the actual concentrations used were 250 ug of dipalmitoyl PC, 250 ug of PE and 500 ug Chol, i.e., some 98.7 % recovery on average.

The ratio of unsaturated to saturated fatty acids in the mixture is shown in table 8.21

TABLE 8.21 UNSATURATED TO SATURATED FATTY ACID RATIO OF TEST LIPOSOME

0.51

An electron micrograph of the test liposome is shown in fig 8.11 and the results of the coagulation testing are shown in fig 8.12. It may be seen that liposomes are formed from the hydration of this mixture of lipids, some multilamellar in form while others are less complex, comprising a unilamellar structure. A variety of intermediate forms is evident.

Fig. 8.11 (opposite) Electron micrograph of vehicle liposomes at a magnification of 32 000. Scale bar is 500nm.

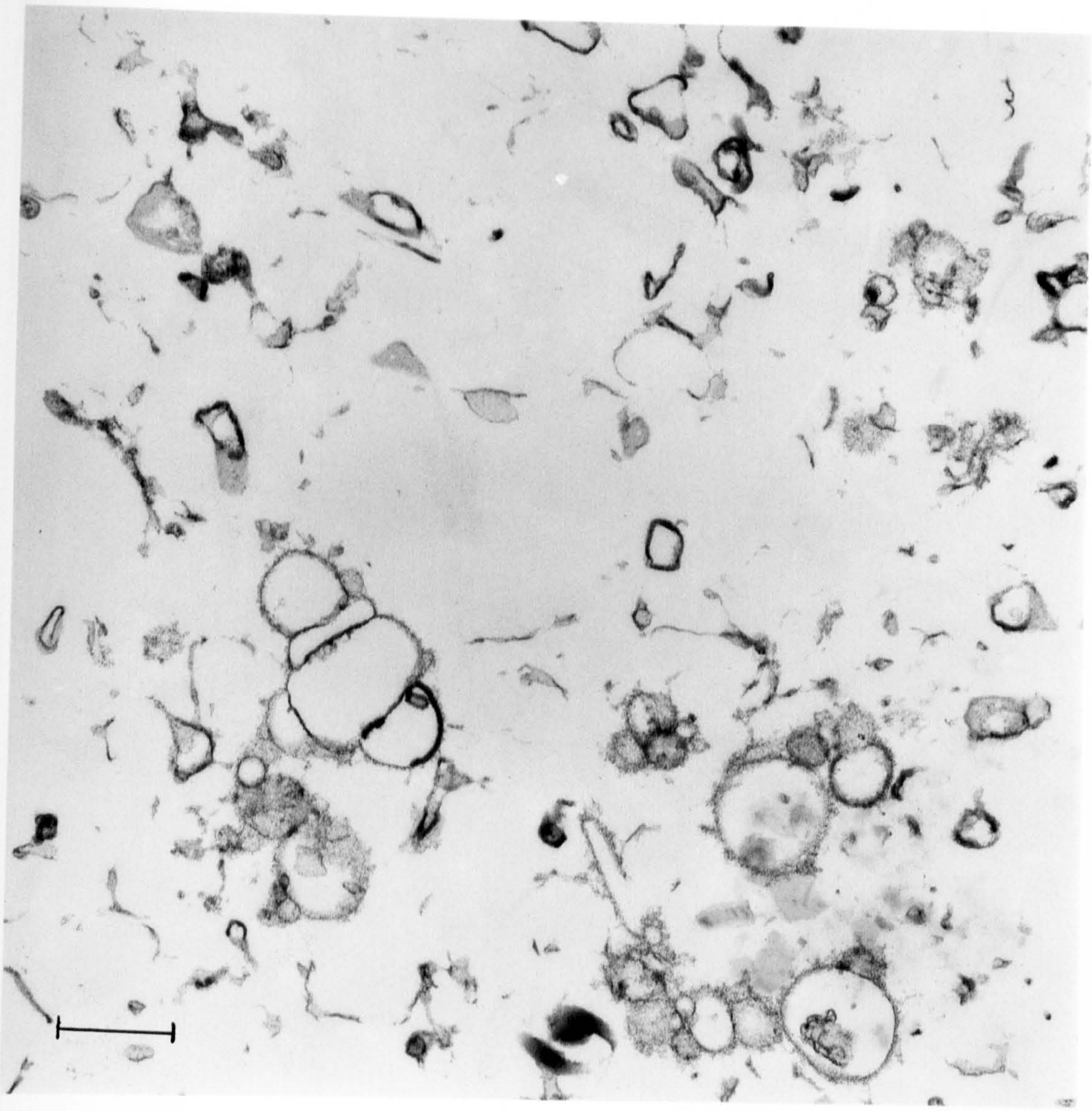


Fig 8.11

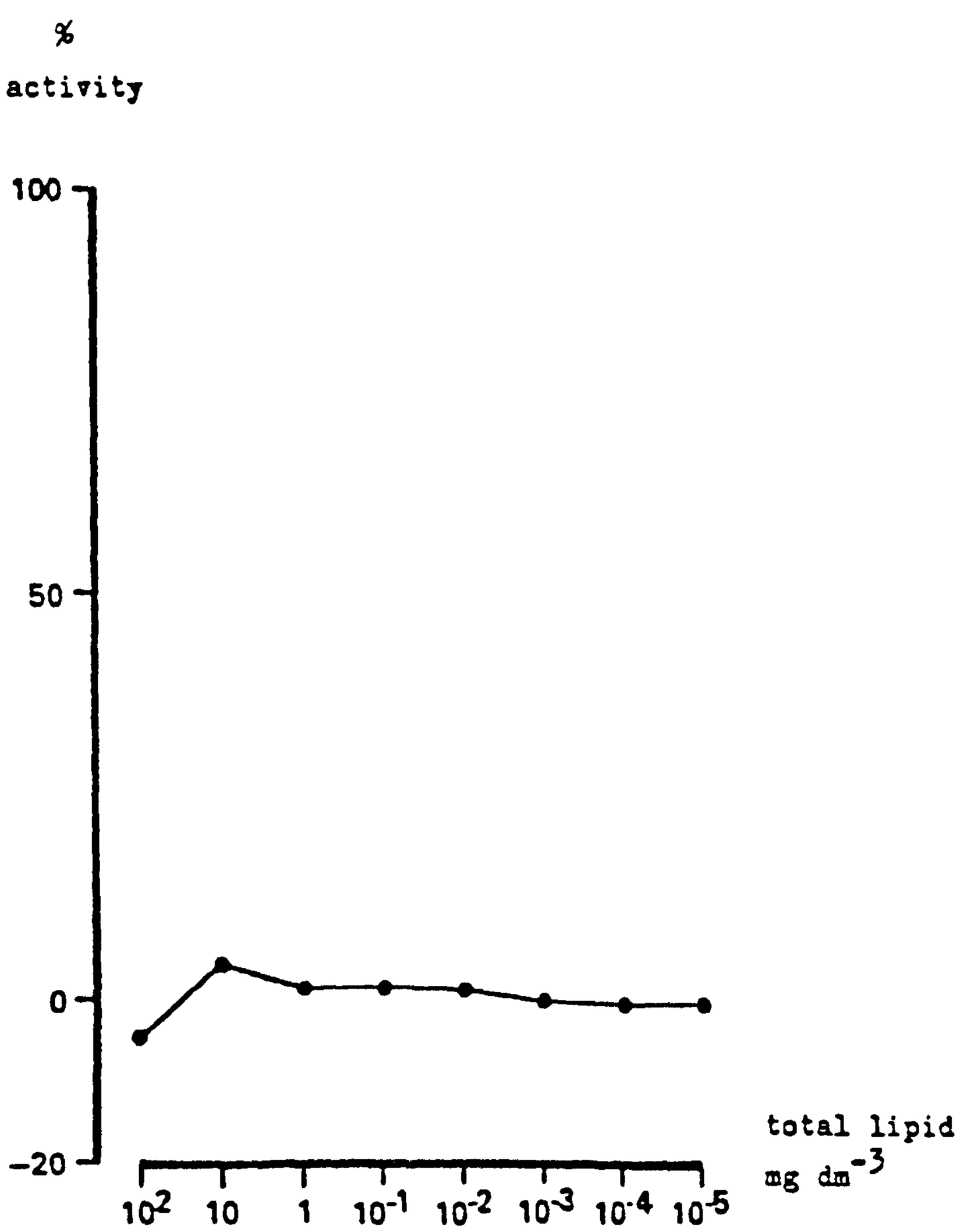


Fig 8.12 Showing lack of activity of the vehicle liposome in the APTT test.

The coagulation testing reveals that there is negligible activity in the liposome, at high lipid concentration, and none whatever at concentrations less than 1 mg total lipid per litre.

Discussion - the test mixture of lipids formed liposomes that had no activity, neither procoagulant nor anticoagulant. Fig 8.11 shows that liposomes were formed, some multilamellar, some unilamellar, with a variety of intermediate forms also present. In testing of clotting activity, no difference was seen between the test mixture and saline control. Several important observations follow. Firstly, liposomes were formed, i.e., a lipid surface was provided and was available in the test. Clearly, this particular surface made no contribution to the APTT test system.

Secondly, a mixture of lipids was employed. Some early experiments, describing the use of individual lipid classes in clotting studies may not have been relevant or realistic since it is unlikely that in any natural system there will be single lipid classes available as clotting surfaces (see Ch 2). The mixture employed here comprised dipalmitoyl PC, chosen because of its saturation and stability, PE from bovine brains because of its degree of unsaturation, and cholesterol. All these components are to be found in natural membranes, although not in these proportions. The combination was useful also since the degree of unsaturation could be subsequently adjusted either by substituting synthetic unsaturated PCs or by adding natural unsaturated phospholipids in to the mixture. Tans et al (1979) have commented

on the relationship between procoagulant activity and lipid phase transitions. They observed that when the phospholipids in their test system were in the liquid phase i.e. the L_α or liquid crystalline state (described in Chapter 2) there was a sharp increase in procoagulant activity. Demel and de Kruyff (1976) showed that introducing cholesterol into layers of PC had a condensing effect on bilayers in the liquid crystalline phase and a liquefying effect on bilayers in the gel state. They found that this effect was complete at cholesterol concentrations of 33 mol % and above. In the experiments described in the present work it was decided to include cholesterol at relatively high concentration so that the lipid mixture employed should be in the "intermediate" state described by Demel and de Kruyff and therefore show no marked procoagulant activity due to altered phase transition. Cholesterol has also been shown to confer structural stability on phospholipid bilayers, e.g. Papahadjopoulos et al (1973 a,b) Blok et al (1975). Inoue (1974) showed that diffusion of a variety of solutes through such bilayers is greatly reduced by incorporation of cholesterol and susceptibility towards phospholipase action is diminished (Op den Kamp et al 1975). Scherphof et al (1979) showed that cholesterol was protective against the plasma-induced solubilisation of multilamellar liposomes at the phase transition temperature. Gregoriadis and Davis (1979) reported a stabilising effect of cholesterol on small unilamellar vesicles made of egg PC and Finklestein and Weissmann (1979) reported on the beneficial effect of cholesterol on liposomal integrity in the presence of plasma or plasma constituents. Cholesterol was added to the lipsomes in this study with the above observations in mind. Thirdly, since the

liposomes formed by this mixture showed no clotting activity this meant that it could be used as a "vehicle" for the testing of other lipid classes.

Conclusion - it proved possible to construct a vehicle liposome with no clotting activity, but with a structure, confirmed by electron microscopy, into which other lipid classes could be inserted for testing of their coagulant properties.

8.8 TO DEMONSTRATE THE PROCOAGULANT PROPERTIES OF THE VEHICLE LIPOSOME INCORPORATING PHOSPHATIDYL SERINE

Aim - to show whether phosphatidyl serine was able to confer procoagulant activity on the vehicle liposome.

Procedure - the vehicle liposome was prepared as in the previous experiment but with the addition of PS, from bovine brain, in a range of concentration i.e. 0, 1.1, 1.6, 1.8, 3.1, 3.75. 6.25, 11.25, 16.95 and 20.4 mol %. Each lipsome was tested over a range of concentration from 100 mg to 10^{-5} mg dm^{-3} of total lipid by measuring the APTT of normal plasma. Procoagulant activity was expressed as % of that of batch 117 of Manchester APTT reagent (see Ch 6.11). The USFAR for each mixture was calculated after gas liquid chromatography of the fatty acid methyl esters on capillary columns (see Ch 6.4).

Results - some examples are given of the performance in the APTT test of the vehicle liposome in the absence of PS and with increasing relative concentration of PS. Fig 8.13a shows the clotting times given when the APTT of a normal plasma was measured using batch 117 (top line) and Owren's buffer (bottom line). The results when 117 was replaced by the vehicle lipsome at various concentrations of total lipid are shown by the broken line. It is clear that the vehicle liposome without PS shows no activity in the test system. Fig 8.13b shows the clotting times when the vehicle lipsome comprises 1.6 mol % of PS. It may be seen that there is now strong procoagulant activity until the total lipid concentration

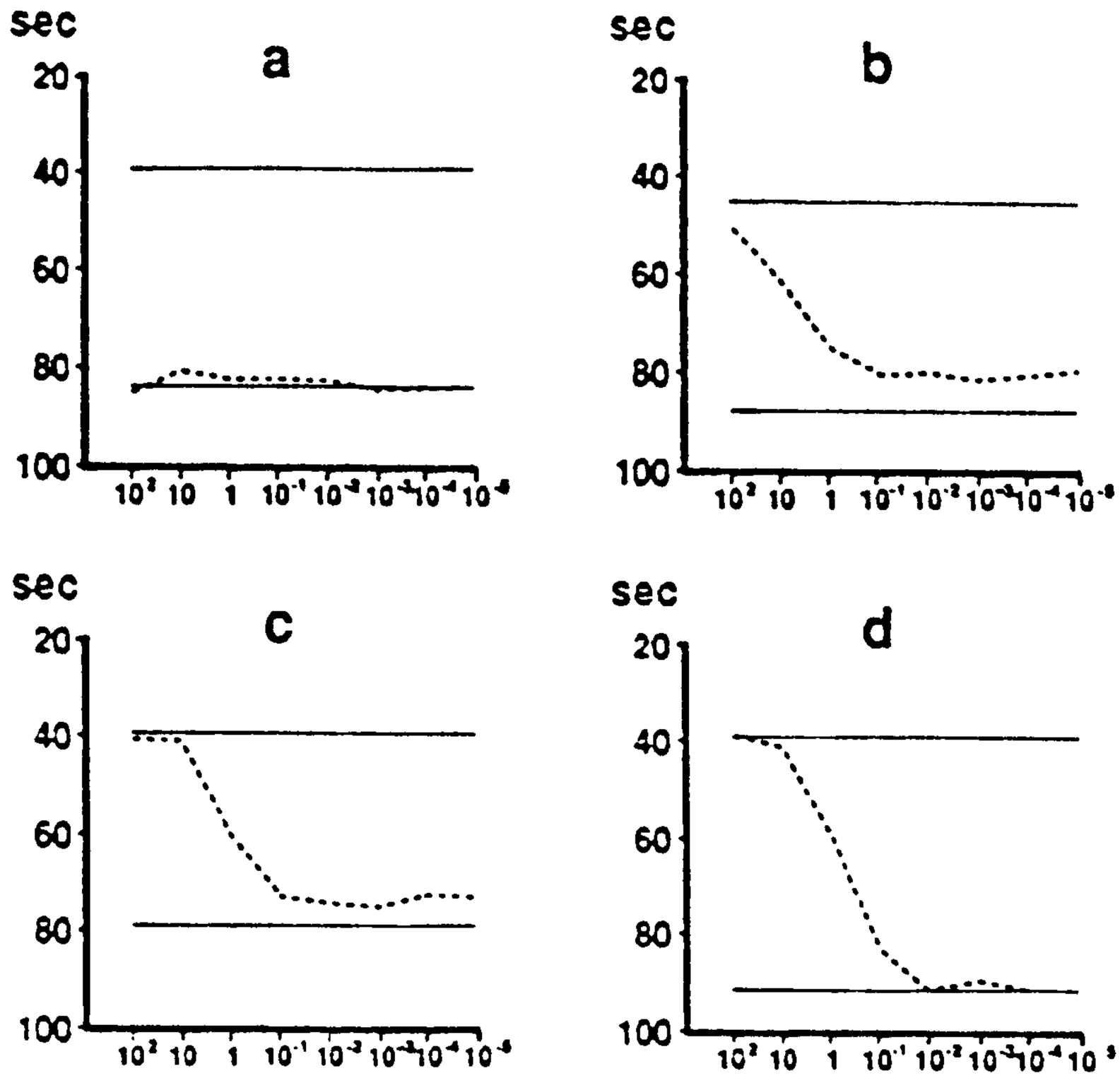


Fig 8.13 Clotting activity of vehicle liposome without (a) and with increasing concentration of PS. b=1.6mol% PS, c=3.1mol% PS, d=6.25mol% PS, e=20.4mol% PS.

falls below 1 mg dm^{-3}. Fig 8.13c shows the performance of the vehicle liposome containing 3.1 mol % of PS. In this clotting time sequence the liposome at 100 mg dm^{-3} had the same activity as batch 117 and showed strong procoagulant activity down to 0.1 mg dm^{-3} of lipid. In Fig 8.13d, the PS level in the vehicle liposome was 6.25 mol %. This liposome had activity comparable to batch 117 at 100 mg and 10 mg dm^{-3} concentrations and had to be diluted to levels of 10 μg dm^{-3} or less before activity was extinguished. Fig 8.13e shows the result of using PS at 20.4 mol % in the vehicle liposome. Again, activity was high at the more concentrated end of the range. The best activity in each instance was shown at concentrations of 100 mg dm^{-3}.

It may be seen that the line indicating the APTT of plasmas in Fig 8.13, when Owren's buffer is used in place of an APTT reagent, is not at the same level in each experiment. The APTT using batch 117 is the same as for the plasma in Fig 8.13a, c and d, which was from the same donor. The Owren's buffer value may differ because there may be some residual platelets or platelet fragments still in the plasma samples following centrifugation. These may accelerate the APTT by providing lipid surfaces.

Fig 8.14 shows a plot of molar concentration of PS against percentage activity in the APTT test. The result for the liposomes containing 100 mg dm^{-3} of total lipid was used. From this plot it is clear that increasing PS concentration increases the procoagulant

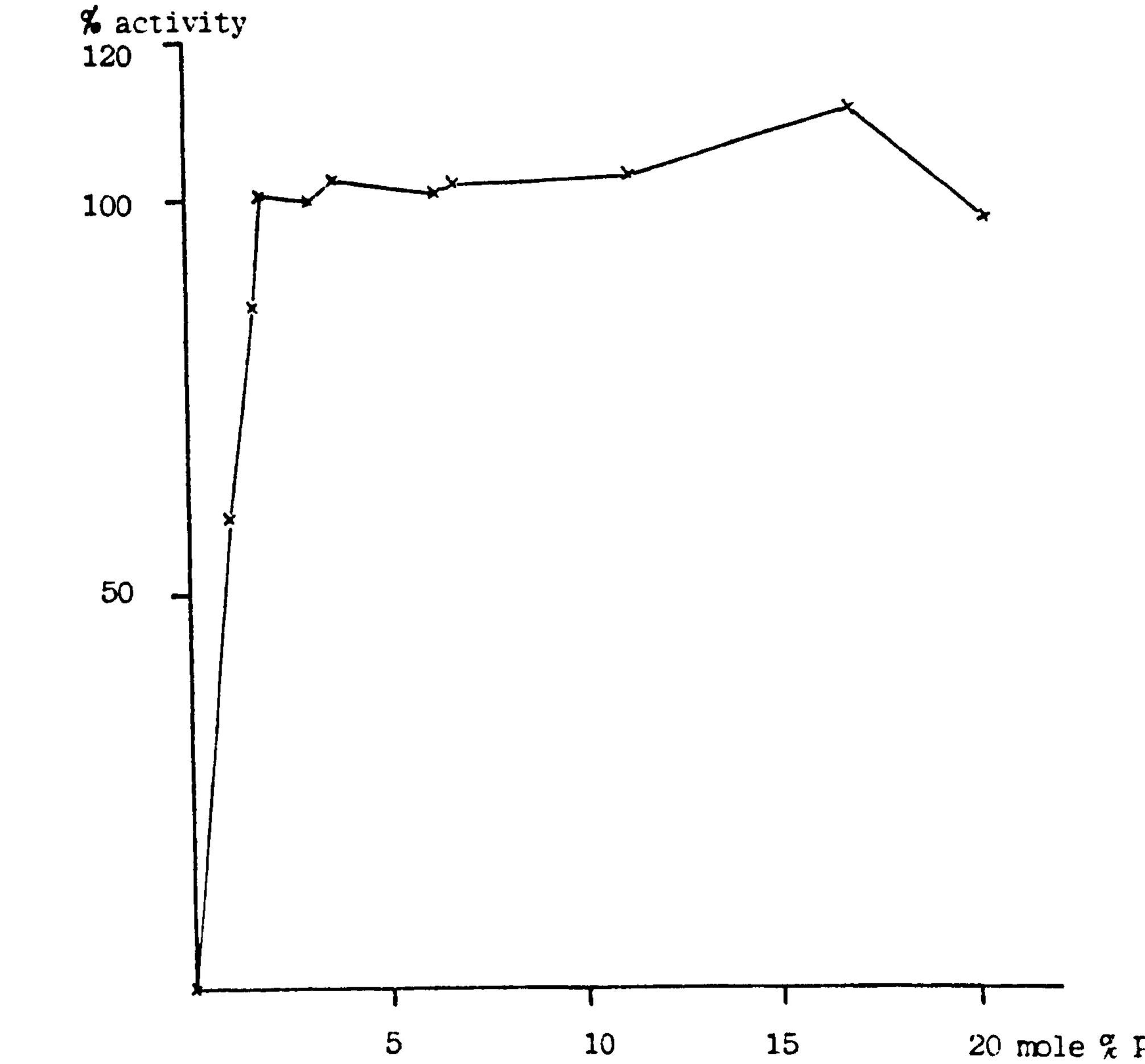


Fig 8.14 concentration of PS vs. activity in the APTT

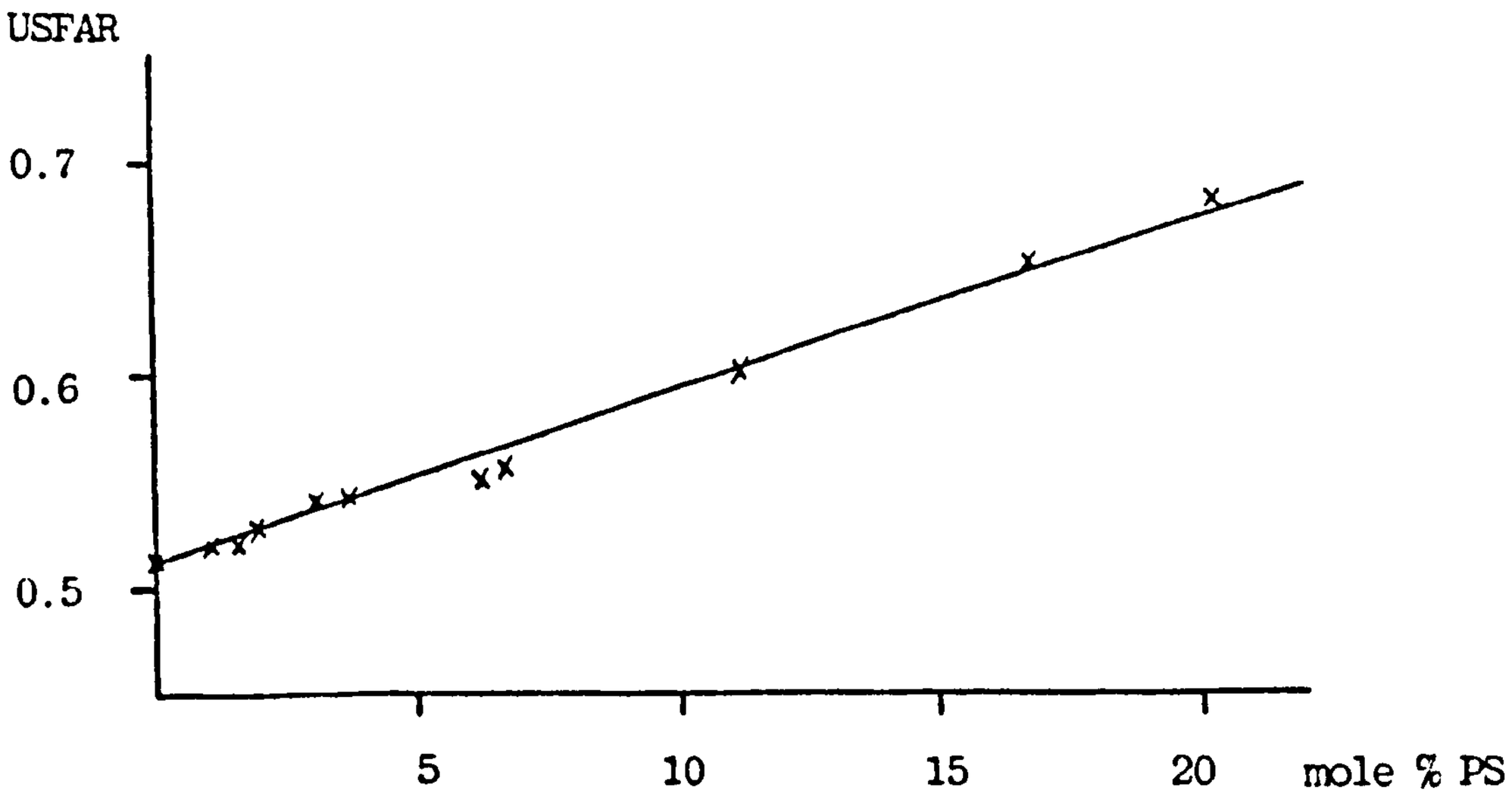


Fig. 8.15 concentration of PS vs. USFAR

activity of the liposomes. The maximum activity is reached when PS is at, or above, a molar concentration of 1.8%.

Fig 8.15 shows a plot of mol % of PS against the USFAR. This plot rises steadily and is not coincident with the activity plot that shows a steeply rising curve followed by a long plateau.

Discussion - procoagulant activity was conferred on the vehicle liposome by the incorporation of PS. This activity reached the equivalent of 100% of that of batch 117 of the Manchester APTT reagent at a molar concentration of 1.8% and thereafter did not significantly alter over the range up to 20.4 mol %. Other workers have commented on the instability of liposomes containing large quantities of PS (Zwaal et al 1978) so it was decided not to proceed beyond this value. The dramatic increase in activity over the narrow range from 0 - 1.8 mol % of PS demonstrates that PS is essential for procoagulant activity of liposomes and that, at low relative concentration, too little is available at the liposome surface for optimal coagulation to take place. At 1.8 mol % there is enough PS available at the liposome surface to provide an optimal environment and there is no advantage in increasing the relative concentration of PS beyond this level. Presumably the available PS on the liposome surface must match the clotting proteins that interact with the phospholipid surface at 1.8 mol %. Above this level PS is in excess. The curve in Fig 8.14 resembles the classical curve of initial velocity versus substrate concentration seen in studies of the kinetics of enzyme-substrate interactions. In this study the requirement for PS in the lipid surface is

analogous to the requirement for substrate in kinetic studies. Above 1.8 mol % PS is in excess and the PS requirement is satisfied.

With the incorporation of PS into the liposome there is a steady increase in the level of unsaturation. While this increase is maintained in a linear fashion with increasing PS concentration, it is clear that the activity of the liposome peaks at the 1.8 mol % PS level. The two curves in Fig 8.14 and Fig 8.15 therefore do not correlate. However, if the first four points on Fig 8.14 are analysed with respect to the first four on Fig 8.15, i.e. the area of steeply rising activity compared with increasing USFAR, a correlation can be found (correlation coefficient is 0.83 $p < 0.05$) which is significant. It may be, therefore, that the level of unsaturation is important in the observed increase in activity.

The differences between the APTT of the plasmas used, both with batch 117 and Owren's buffer, is of interest. Complete removal of platelets from the plasma would have been desirable but may not have been achieved entirely. This would account for the differences seen at the Owren's buffer end of the range. However, by using the batch 117 results as 100% and the Owren's buffer results as 0% activity, it was possible to compare the activities of the liposome preparations on different days and with different plasmas.

Conclusions - the incorporation of PS into the vehicle liposome rendered it active in the APTT test system, with concentrations of 1.8 mol % and above providing a favourable catalytic surface for the coagulation proteins. The level of unsaturation is unlikely to be

the key determinant of the rapid increase seen in the activity curve.

It was possible to construct a liposome, made from individual pure lipids, which showed the same procoagulant activity as batch 117 of Manchester APTT reagent, an extract of human brain lipids.

8.9 TO DETERMINE THE COAGULANT PROPERTIES OF A PS-CONTAINING LIPOSOME UPON DILUTION

Aim - to show the effect of dilution on the performance of a PS-containing liposomal suspension in the APTT test.

Procedure - a liposome was prepared of the following composition - dipalmitoyl phosphatidyl choline, phophatidyl ethanolamine, phosphatidyl serine and cholesterol at 16, 16, 3 and 65 mol % respectively. It was tested in the APTT system over the range 100 mg to 10^{-5}mg dm^{-3} of total lipid.

Result - Fig 8.16 shows the dilution curve of the PS-containing liposome. No anticoagulant activity was demonstrated at any concentration tested.

Discussion - the PS-containing liposome showed procoagulant activity over the entire range of concentration tested. Since the liposome under study was made up of four lipid classes, each individually checked for purity (see Chapter 6.7), it is reasonable to wonder whether some early work which reported PS to have anticoagulant activity, may have employed lipids that were not pure, in systems where liposomes were not formed, i.e., where "membranes" were not provided for the interaction of the clotting sequence (Marcus et al 1962, Troup and Reed 1958, Turner and Silver 1963, Mustard et al 1962). This point is discussed further (see section 8.14).

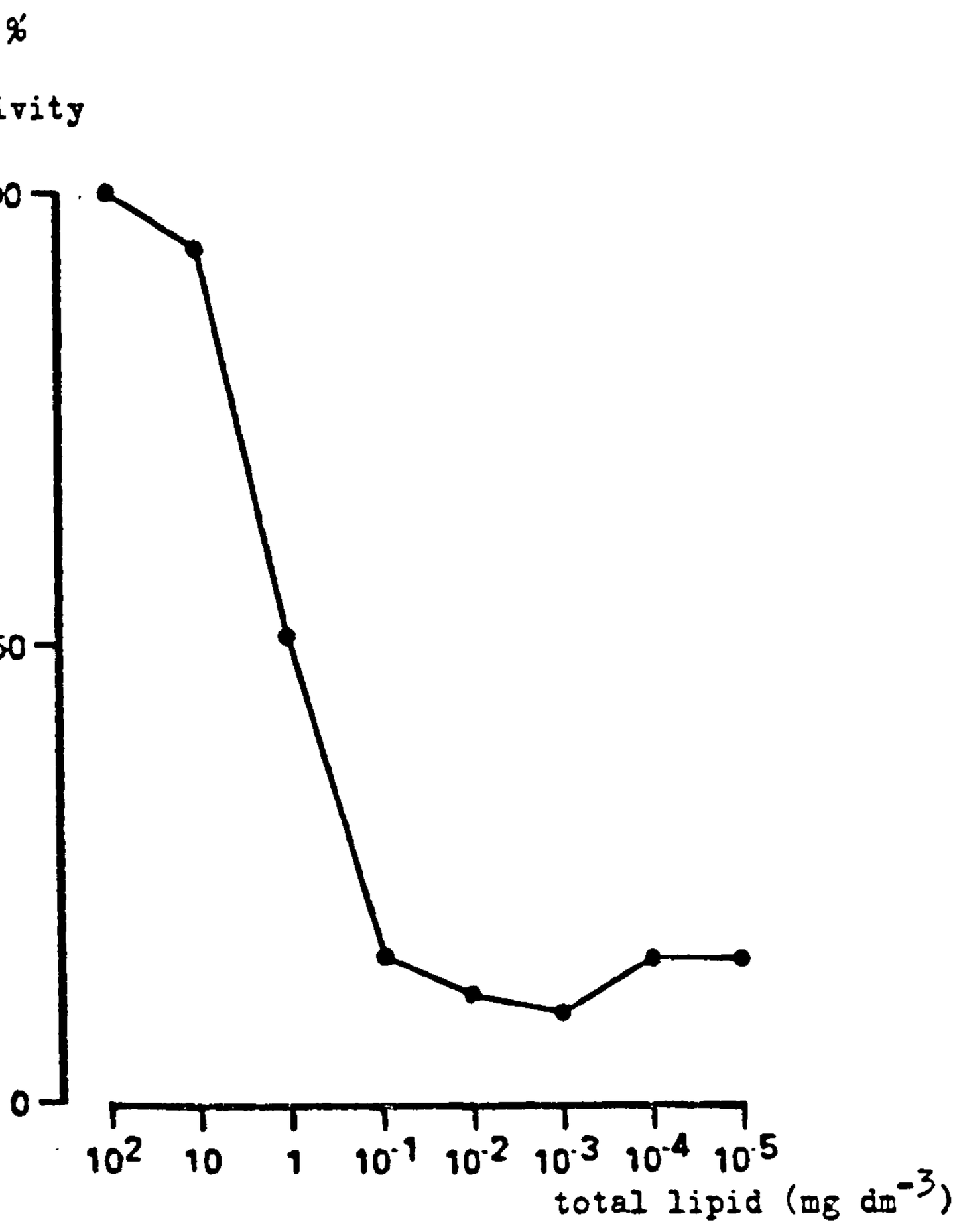


Fig 8.16 Dilution curve of PS-containing liposome.

The "dilution curve" approach employed in this experiment proved useful as a device for characterising the coagulant activity of a liposome suspension.

8.10 TO DETERMINE WHETHER THE PROCOAGULANT ACTIVITY OF PS MAY BE REPLACED BY LYSO-PS, PG OR PI

Aim - to test whether lyso-PS, PG or PI, phospholipids which, like PS, possess negative charge, will substitute for PS in the test mixture.

Procedure - a liposome identical to that described in experiment 8.7 was prepared. This was arbitrarily designated the "standard" preparation. Four other liposomes were prepared according to the following table

TABLE 8.22 COMPOSITION OF TEST LIPOSOMES

1.	DPPC	:	PE	:	lyso-PS	:	CHOL	
	15.42		16.52		5.07		62.99	
2.	DPPC	:	PE	:	PG	:	CHOL	
	15.7		16.8		3.36		64.13	
3.	DPPC	:	PE	:	PI	:	CHOL	
	15.77		16.89		2.93		64.41	
4.	DPPC	:	PE	:	PS	:	PI	: CHOL
	15.75		16.87		1.57		1.46	64.34

Each liposome mixture was tested by preparing a dilution curve, as in experiment 8.7. The same range of concentration was used i.e. 100 mg to 10^{-5} mg dm^{-3}.

The USFAR was determined by gas liquid chromatography of the methyl esters of the liposome fatty acids (see Ch 6.4).

Results - the coagulant activity test results are given in Fig 8.17. Lyso-PS showed maximum procoagulant activity in the liposome suspension containing 100 mg dm^{-3} of total lipid. This activity, about 20% of batch 117, fell as total lipid reduced.

PG showed no activity whatsoever in this test system - neither procoagulant nor anticoagulant. PI - containing liposomes at high total lipid concentration showed about 45% of the activity of batch 117. Upon dilution, procoagulant activity was lost, to be replaced by a slight anticoagulant effect at concentrations of total lipid below 100 μg dm^{-3}. When PS was replaced by a mixture of PS and PI, procoagulant activity was evident at high total lipid concentration (equivalent to 96% of the activity of batch 117). A quite dramatic anticoagulant effect was seen at lower total lipid concentrations, however. This effect was much more marked than in the liposomes that contained PI alone. USFAR values are shown in Table 8.23.

TABLE 8.23 UNSATURATED TO SATURATED FATTY ACID RATIOS OF THE FOUR TEST LIPOSOMES

liposome	USFAR
1	0.49
2	0.58
3	0.57
4	0.56

These values were close to that of the standard lipsome i.e. 0.54 (see Ch 8.11) and there was no correlation between USFAR and activity in the APTT system.

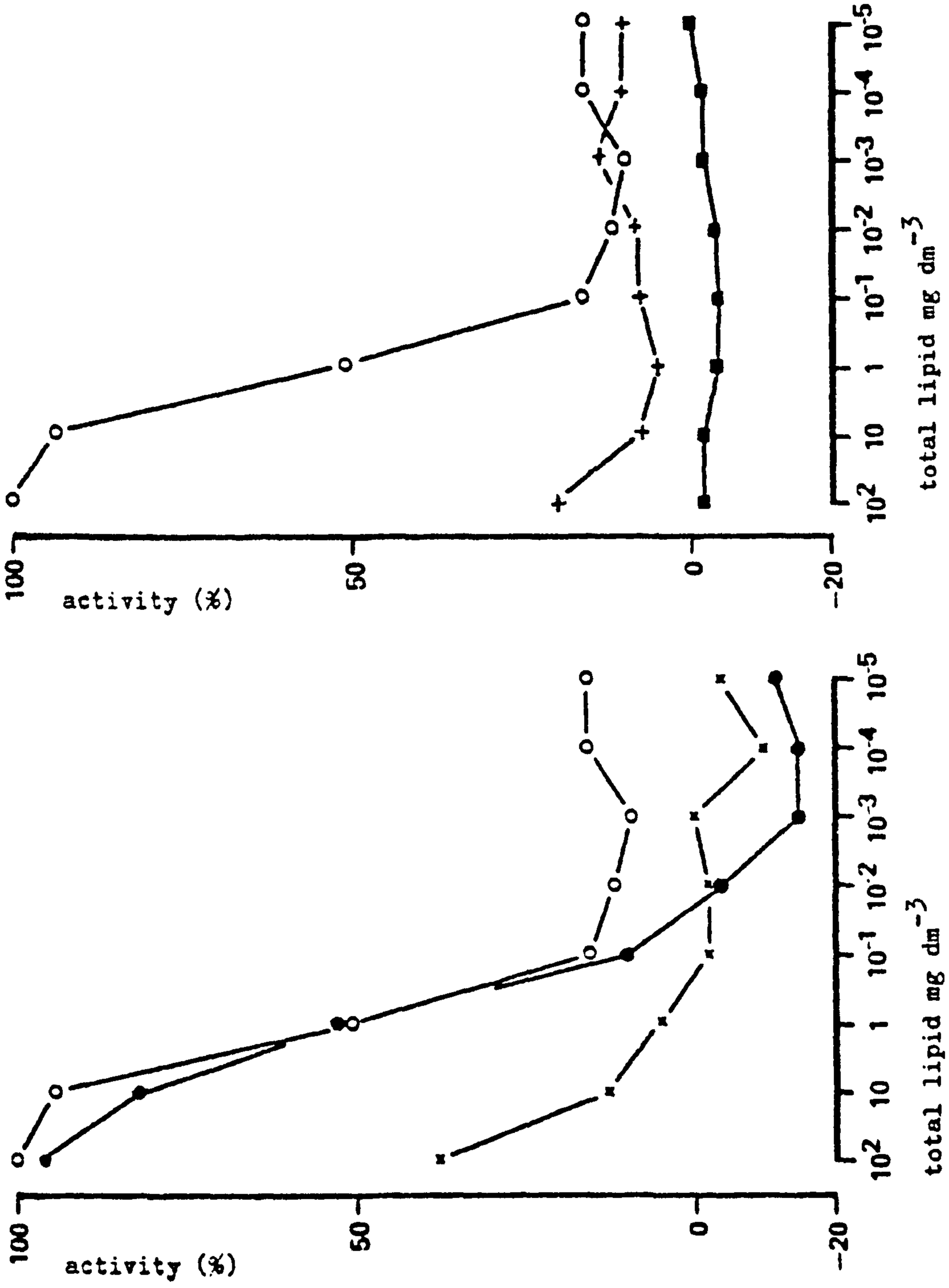


Fig 8.17 Clotting performance of liposomes containing PI (x——x) PI+PS (●——●), lyso-PS (+——+), and PG (■——■) compared with a standard liposome containing PS (o——o).

Discussion - PS was unique in conferring procoagulant character in this test system. Lyso-PS with the identical head group and therefore the same net charge, could not replace its activity. PG was inert, which was a useful observation since it proved possible to use PG as a make-weight" in subsequent studies.

The role of PI proved to be somewhat perplexing. Fig 8.17 showed that PI in the vehicle liposome does exhibit some procoagulant activity, although at best less than half that shown by PS. The combination of PS and PI together showed good procoagulant activity at high concentration (some 96% of that shown by PS alone) but a marked anticoagulant effect at low total lipid concentration. This anticoagulant effect was more marked than for PI alone.

Bevers and Zwaal (1988) reported that SPH and PE have a modulating effect on the prothrombinase and tenase activities at the activated platelet surface. In this present experiment it is clear that PI has some regulatory effect also.

Billah and Lapetina (1983) observed that PI concentration reduced upon platelet activation and subsequently rose to higher levels than in the resting platelet. This implies that PI has some inhibitory function. On the other hand it has been reported that PI is almost exclusively found on the cytoplasmic side of the platelet membrane (Chap et al 1977, Zwaal et al 1977, Zwaal 1978, Perret et al 1979, Schick 1979). This implies that it is procoagulant and is located away from the plasma clotting protein in the same way as PS. In vivo, PI metabolism is under control of a series of

reactions known as the PI cycle. Splitting of phosphatidyl-4,5-bisphosphate, (PIP2) by an endogenous phospholipase C (PLC) (Lapetina and Cuatrecases 1979, Bell and Majerus 1970) gives rise to inositol-1,4,5-triphosphate and diacylglycerol (DG). The former loses phosphate groups, one at a time, until myo-inositol remains. Meanwhile, DG is phosphorylated to form PA and PI is formed following an intermediate step involving cytidine diphosphate. Several important features occur in this cycle, e.g., DG activates protein kinase C, leading to phosphorylation of a protein, of 47,000 daltons molecular weight, that is essential in the secretion response (Siess et al 1983). Arachidonic acid is freed from DG by a PLC that is less Ca^{2+}-dependent than the phospholipase A2 (PLA2) that splits arachidonic acid from PC and PE (Simon et al 1984). This reaction may free small amounts of arachidonic acid while cytoplasmic Ca^{2+} is at low concentration, until adequate Ca^{2+} concentration is achieved for stimulation of PLA2, which generates the major part of the free arachidonic acid. Cyclooxygenase and thromboxane synthetase enzymes, contained in platelet dense tubular system membranes are able to oxidise arachidonate to form endoperoxides and thromboxanes. Thromboxane A2 is a potent platelet activator. PA, meanwhile, can act as a Ca^{2+}-ionophore and aid the influx of extracellular Ca^{2+}, which stimulates the release of arachidonate by PLA2 and activates contractile activity by an effect on myosin light chain kinase (Feinstein et al 1981, Hallam et al 1984). These are some of the in vivo events of the PI cycle. In the present experiment, the plasma used was essentially platelet-free so none of these can have taken place. Instead, PI will have been at a relative high, possibly

inhibitory concentration. This was clear at the highest concentration of total lipid, when the procoagulant activity was 96% that of batch 117 and even more marked at low total lipid levels when strong anticoagulant character was seen.

It may be that there was sufficient PS available in the procoagulant liposome to override any inhibitory effect of PI. On dilution this effect became more marked. PI will have interfered with the packing density of PS in the liposome any may therefore alter the surface arrangement so that protein binding is inefficient. It is tempting to speculate that the "conformation model" of binding described by Forman and Nemerson (1986) and van Rijn et al (1986) may apply in this system and that the binding to the PI containing liposome induces a conformational change in the clotting enzyme that results in a molecule less able to bind soluble substrate.

It has been suggested that negative charge is crucial in conferring procoagulant activity on a lipid mixture (Bangham 1961, Papahadjopoulos 1962, Daemen et al 1965, Marcus 1966, Bull et al 1972). If this was all that was essential for expression of procoagulant activity then it should have been possible to replace PS by other phospholipids of negative charge.

Conclusions - PS was unique in its ability to confer procoagulant activity on a liposome mixture. Lyso-PS, PG and PI could not substitute for PS in this test system. PG was completely

inert. This meant that it could safely be used as a "make-weight" in further studies.

In certain circumstances, PI expressed procoagulant or anticoagulant character. In concert with PS, both these effects were greatly amplified.

Finally, the difference in USFAR between the four test liposomes was not sufficient to explain the wide disparity in procoagulant properties observed. Differences in polar head group, charge, or in packing density due to fatty acyl composition may be more likely. This is considered in greater detail in Chapter 8.14.

8.11 TO DETERMINE THE EFFECT OF VARYING THE USFAR OF TEST LIPOSOMES

Aims - to observe whether there are any changes to the dilution curves of liposomes prepared with widely differing levels of unsaturation.

Procedure - the standard liposome was prepared as described above. Two more saturated liposome mixtures were prepared by substituting a synthetic dipalmitoyl PE for the natural PE of the standard mixture. A more unsaturated mixture was prepared by substituting dioleyl-PC for the dipalmitoyl PC of the standard mixture. Four types of liposomes were prepared in all with compositions as shown in table 8.24.

TABLE 8.24 COMPOSITION OF TEST LIPOSOMES

1.	DPPC	:	DPPE	:	PS	: CHOL
	39		39		8	14
2.	DPPC	:	DPPE	:	PS	: CHOL
	15		15		7.5	62.5
3.	DPPC	:	PE	:	PS	: CHOL
	16		16		3	65
4.	DOPC	:	PE	:	PS	: CHOL
	14		5		17.3	65.5

USFARs for these preparations were determined by GLC as described (Ch 6). Each liposome mixture was tested as described above and a dilution curve constructed.

Results - Table 8.25 shows the USFARs for the four liposome mixtures.

TABLE 8.25 USFAR OF LIPOSOME MIXTURE

mixture no.	USFAR
1	0.04
2	0.10
3	0.54
4	3.67

Figure 8.18 shows the result of testing the coagulant properties of the four liposome mixtures. At high total lipid concentration there is little difference between preparations 2, 3 and 4. Preparation no. 1, the most saturated, had less procoagulant activity than the others at each concentration tested. It was not until total lipid concentrations of 100 ug dm^{-3} or less were tested that further differences became apparent. The most striking observation was the strong anticogulant character of liposome 1, the most saturated mixture. Liposome 2 also showed some anticoagulant activity. Liposomes 3 and 4, which behaved in closely similar fashion at high total lipid concentration, had markedly different coagulant character at low lipid concentration, with liposome 4, the most unsaturated preparation, showing higher procoagulant activity in this area.

Discussion - the degree of unsaturation of membrane fatty acids has been shown to influence their clotting activity e.g. Ploplis and Castellino (1980), Tans et al (1979). In this experiment four-component liposomes were made and unsaturation adjusted by substituting more or less saturated phospholipids into the mixtures. The fatty acyl component of the phospholipids was altered

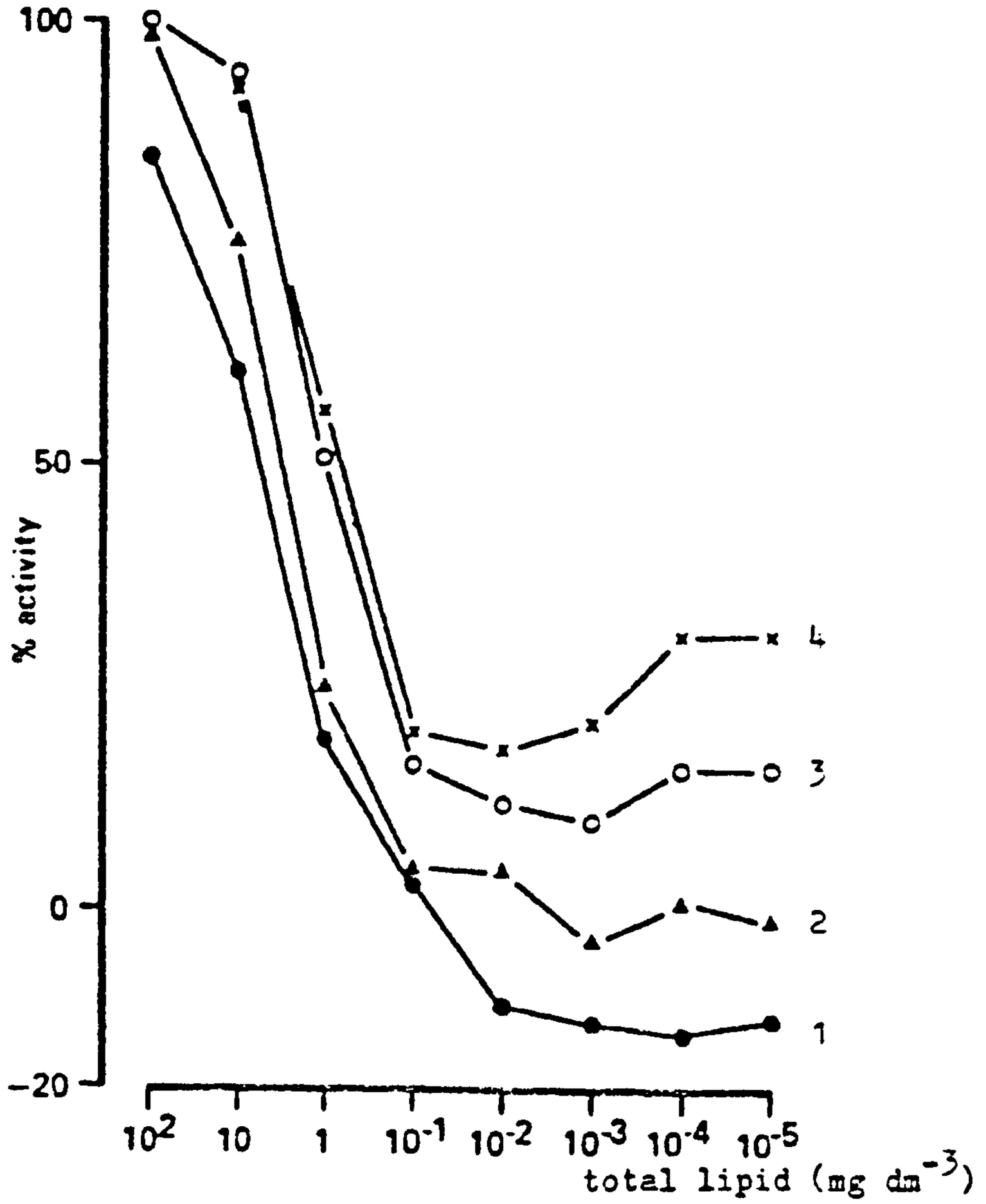


Fig 8.18 Coagulant activity of liposomes of varying USFAR. 1=0.04, 2=0.10, 3=0.54, 4=3.67

as shown in table 8.24 with resultant change in the USFAR, shown in table 8.25. The overall trend, even though there was some change in the relative concentration of PS, was that the most saturated mixture was the least procoagulant at high lipid concentration.

The relative concentrations of PS were within the range that gave optimal activity in the experiments described in Chapter 8.8 (see Fig 8.14) i.e. 1.8 mol % to 20.4 mol %. PS should therefore be present in sufficient quantity.

The most unsaturated mixture contained the highest procoagulant activity at every concentration tested. Changes in PS concentration were unlikely to account for this trend. The density of packing of the PS molecule in the liposome is more likely to be the determining factor - dense packing, as would be possible in a relatively saturated liposome where the fatty acyl cross-sectioned area is smaller than that in an unsaturated fatty acid environment (Lee 1975, Mean 1986) would present an unfavourable surface for reaction. In the more unsaturated liposomes, the packing density will be reduced, with the result that PS is available in optimal configuration for interaction with the clotting proteins (Zwaal and Hemker 1982). There is really very little difference, in practice, between liposomes 3 and 4 at total lipid concentrations between 100 mg and 0.1 mg dm^{-3} despite the substantial difference in USFAR.

As seen in Chapter 8.4, the major APTT reagents have total lipid concentrations of 0.33 to 75.31 µg per test. In the present experiment 100 mg dm^{-3} was the highest concentration tested. This is equivalent to 10 µg per test. This preparation was then diluted

ten-fold at each stage of testing so that by the time the 10^{-1} mg dm^{-3} concentration was reached there was already less lipid present than in any of the commonly used APTT reagents.

Conclusions - at high total lipid concentration there is little difference between the four test liposome preparations. Upon dilution, differences become apparent and the more unsaturated preparations retain procoagulant activity to lipid concentrations of 10^{-5} mg dm^{-3}, while the saturated preparations show some anticoagulant character at this low concentration.

The work described in sections 8.7 to 8.11 formed the basis for a paper entitled "The procoagulant activity of partial thromboplastin extracts: the role of phosphatidyl serine" published in Thrombosis Research Vol 26 pp 341-350 (1982). A reprint of which is appended.

8.12 TO EVALUATE THE ROLE OF PHOSPHATIDYL SERINE IN CONFERRING SENSITIVITY TO HEPARIN ON THE APTT TEST

Aim - to evaluate the effect of alterations in liposome mixtures used in the measurement of heparin by the APTT.

Procedure - liposomes were prepared comprising di-palmitoyl PC, PE and Cholesterol. PS was incorporated in a range from 3 to 18 mol %. In order to maintain a constant total lipid concentration, phosphatidyl glycerol was added to bring the total lipid concentration to 100 mg dm^{-3}. PG was observed to have no activity in an earlier experiment (Expt 8.10). In a further series of liposome mixtures, PI was substituted for PS, to test for any activity in this system. In order to test the effect of reducing the total lipid available in the test system, liposomes were prepared at total lipid concentrations of 1000, 100, 50, 30, 20 and 10 mg dm^{-3}. In each liposome the relative properties of the components were kept constant. PS concentration was 10.8 mol %. Coagulation testing was by using the liposome preparations instead of the Manchester APTT reagent in determining the APTT of fresh normal plasma before and after the addition of a range of concentrations (i.e. 0.05 u cm^{-3} to 0.2 u cm^{-3}) of British Standard Heparin. A sensitivity ratio i.e. APTT of plasma with heparin/APTT of plasma without heparin was derived for each heparin concentration studied. The USFAR for each liposome mixture was determined by GLC.

Results - as the relative concentration of PS in the test system increased the APTT of plasma, with and without heparin, decreased as shown in Fig 8.19. The heparin sensitivity ratio was decreased as a result with increasing concentrations of PS (see Fig 8.20).

When PS was replaced by PI the APTT was markedly increased. Increasing the relative concentration of PI, however, did not decrease the APTT. The heparin sensitivity ratios showed no marked change with increasing concentrations of PI (see Figs 8.21 and 8.22). Table 8.26 lists the USFAR of the mixtures containing PS and PI. The ratio of unsaturated to saturated fatty acids decreased with increasing PS and PI concentration. This was because the PG incorporated as makeweight had a higher level of unsaturation than either the PS or PI.

Alteration of the total lipid concentration from 1 g dm^{-3} to 100 mg dm^{-3} produced no change in either APTT or heparin sensitivity ratio, whereas the tests performed with 10 mg dm^{-3} of lipid increased the APTT and the sensitivity ratio, as shown in Figs 8.23 and 8.24. The observation became more marked as the concentration of heparin increased. Figs 8.25 and 8.26 show that by testing a further range of total lipid concentration of 100, 50, 30, 20 and 10 mg dm^{-3}, the APTT and the heparin sensitivity ratios only increased below a total lipid level of 30 mg dm^{-3}.

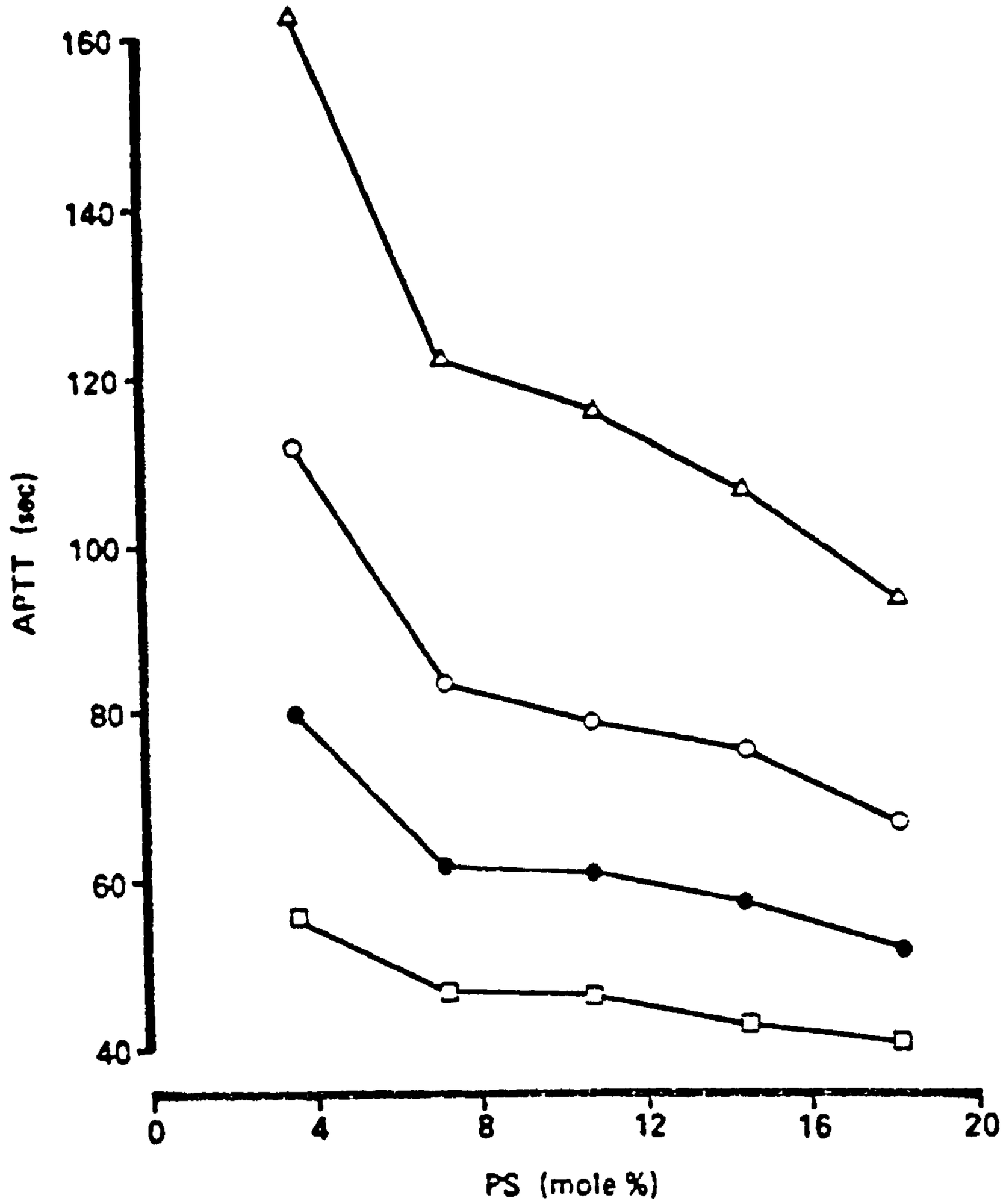


Fig.8.19 effect of increasing PS on APTT of heparinised plasma

□—□ = plasma with no additions

●—● = plasma + 0.05 u cm^{-3} heparin

○—○ = plasma + 0.10 u cm^{-3} heparin

Δ—Δ = plasma + 0.20 u cm^{-3} heparin

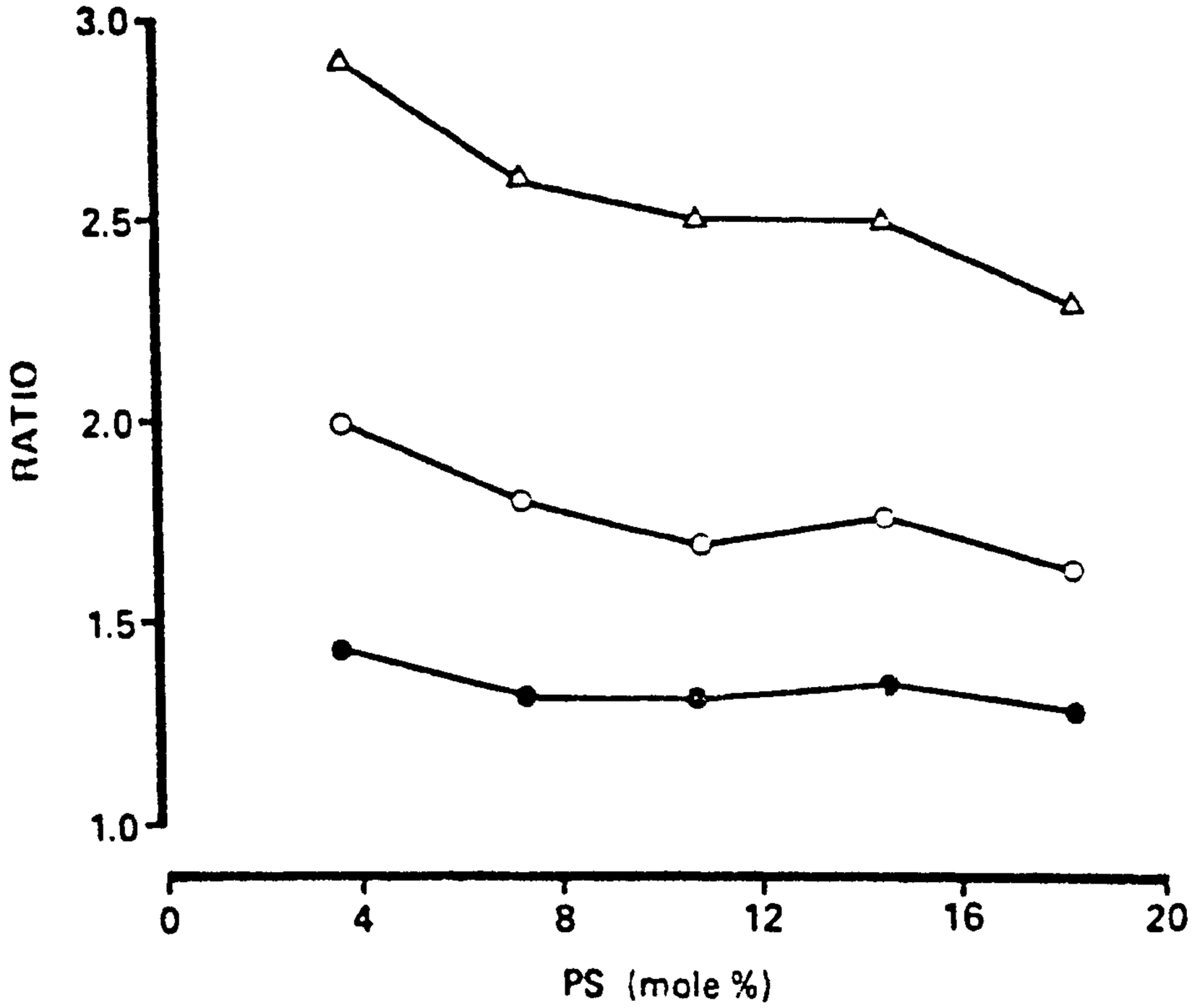


Fig 8.20 Effect of increasing PS concentration on the heparin sensitivity ratio. Symbols as in Fig 8.19

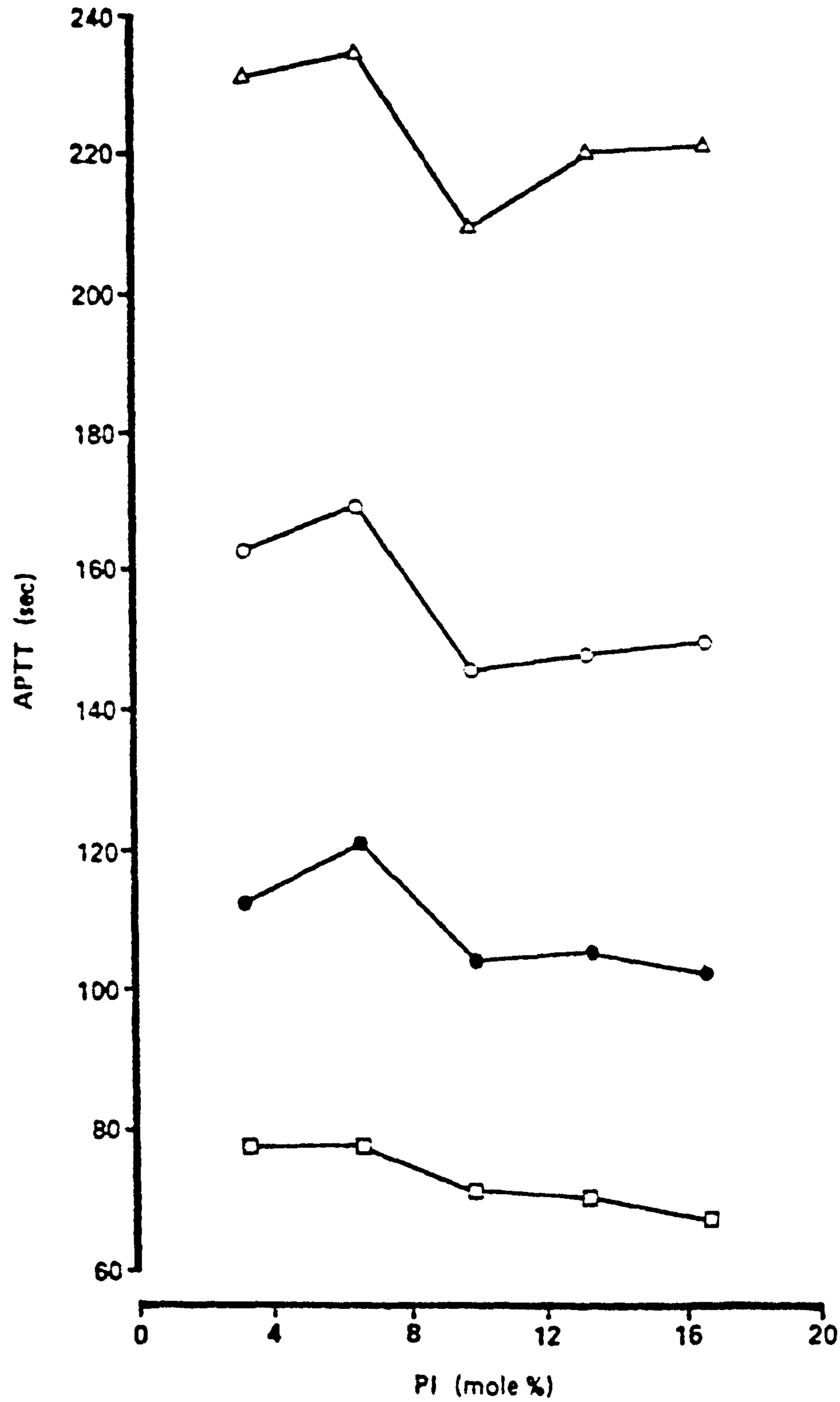


Fig 8.21 Effect of replacing PS with PI on the APTT of heparinised plasma. Symbols as in Fig 8.19

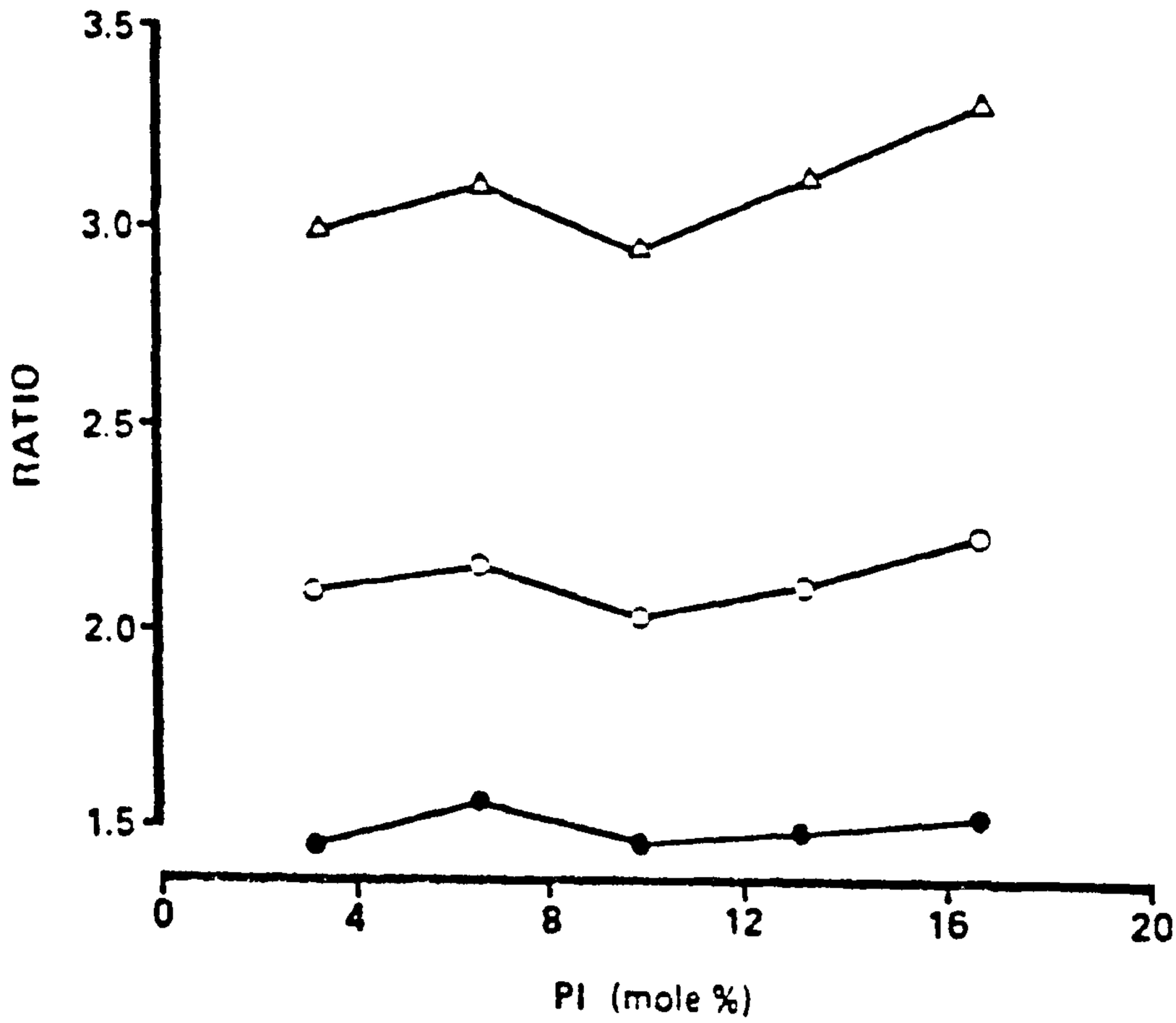


Fig 8.22 Effect of replacing PS with PI on the heparin sensitivity ratio. Symbols as in Fig 8.19

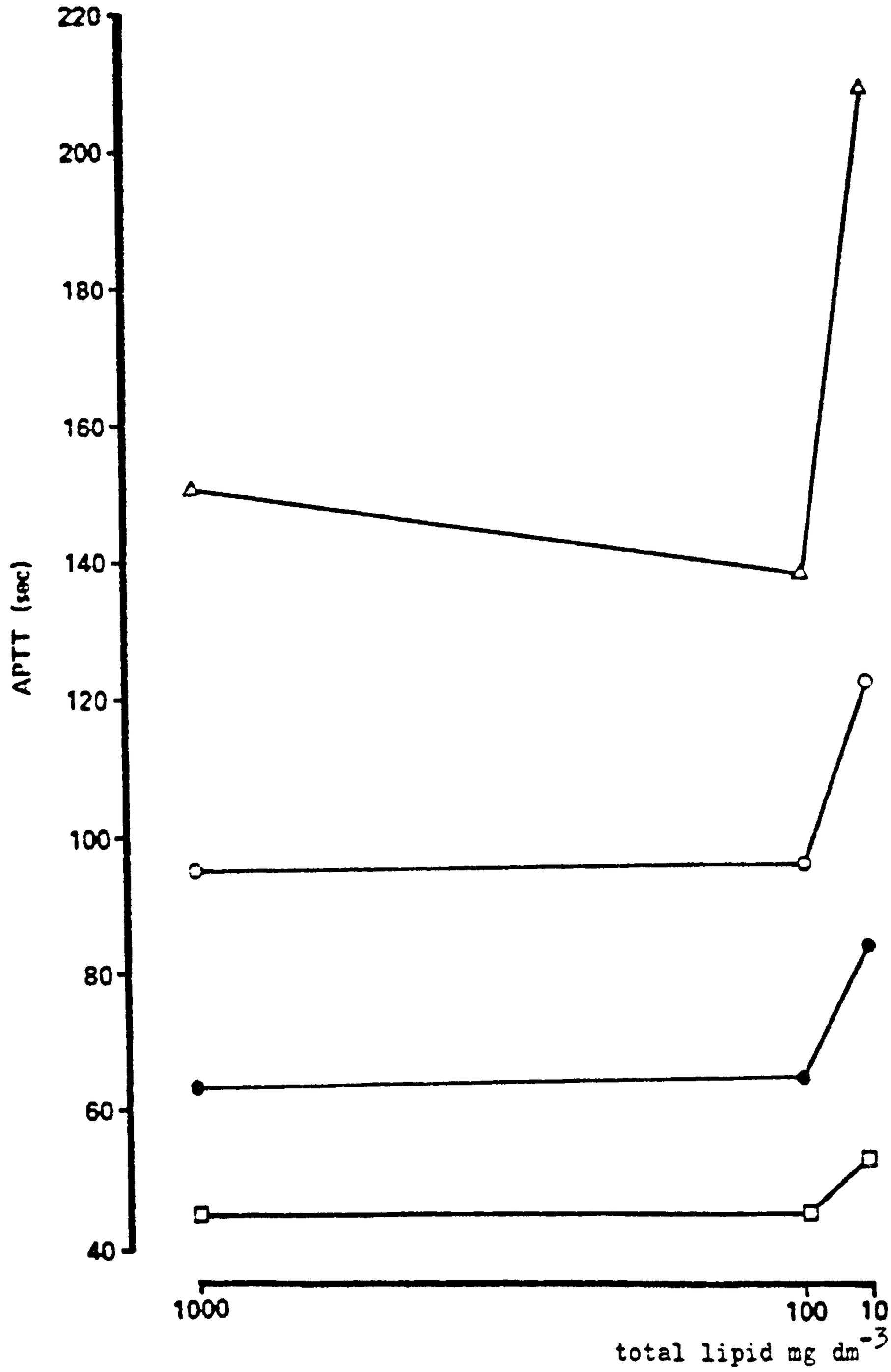


Fig 8.23 Effect of decreasing total lipid on APTT of heparinised plasma. Symbols as fig. 8.19.

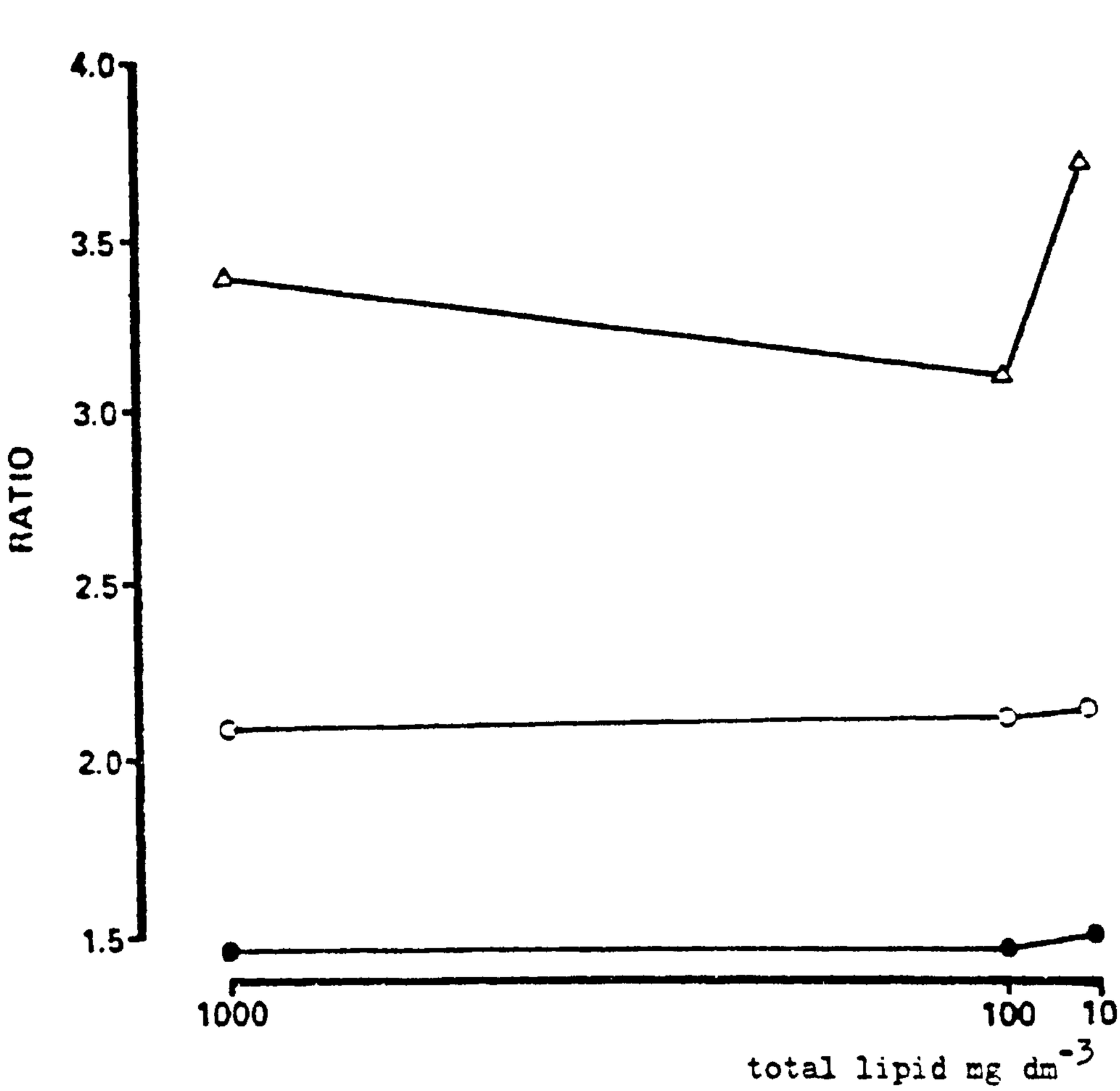


Fig. 8.24 Effect of decreasing total lipid on heparin sensitivity ratio. Symbols as fig. 8.19.

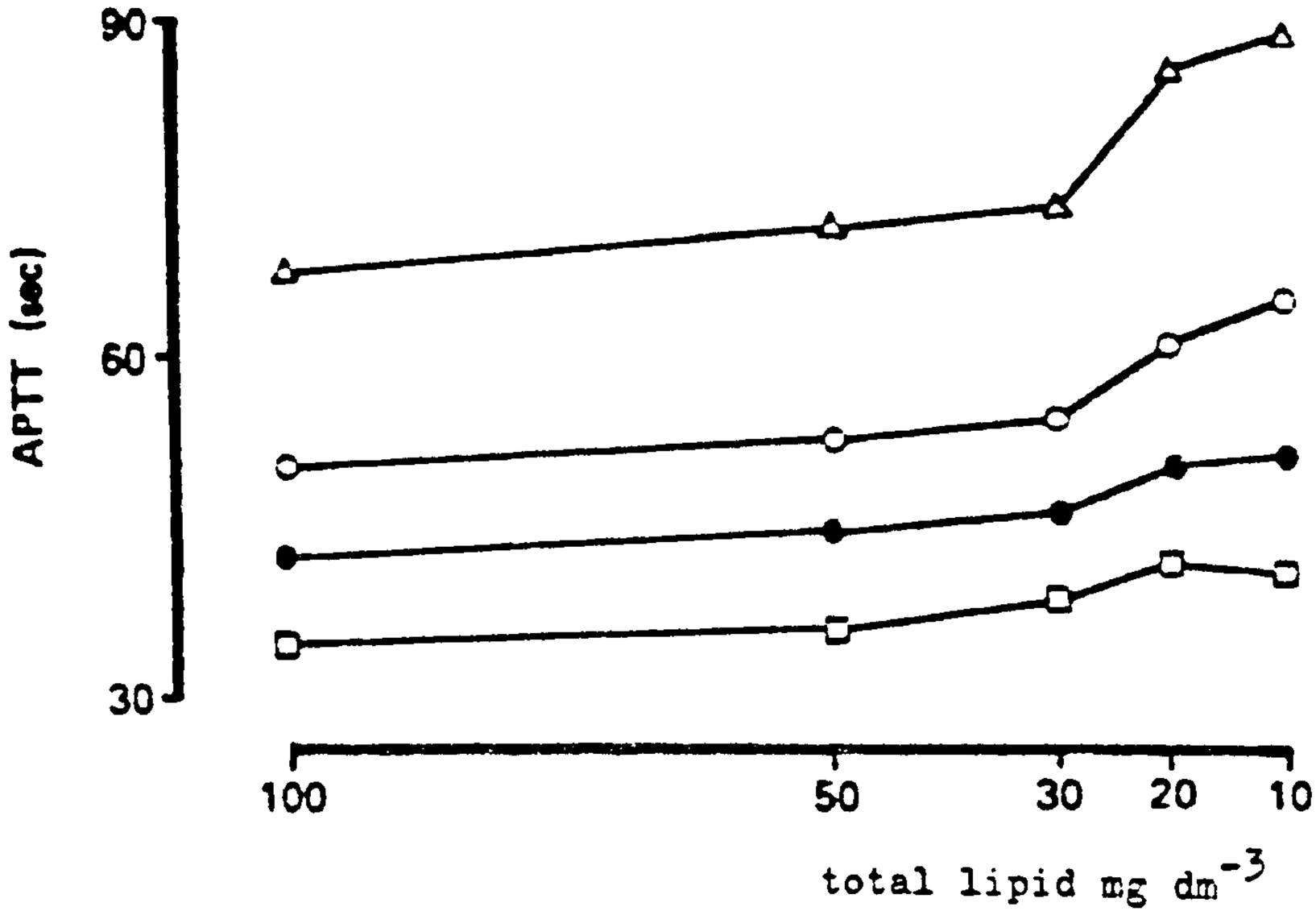


Fig. 8.25 Effect of decreasing total lipid on APTT of heparinised plasma. Symbols as fig. 8.19

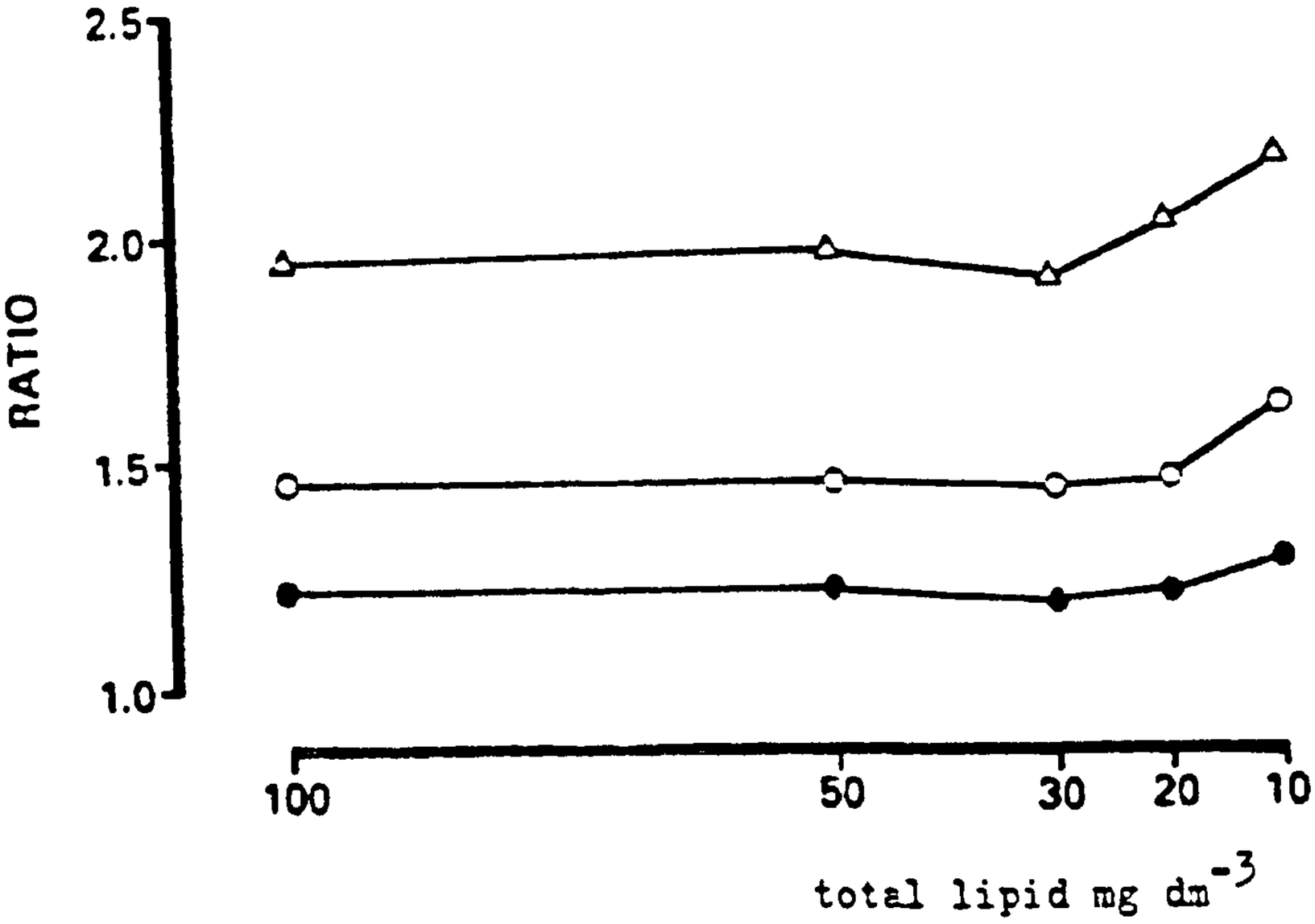


Fig 8.26 Effect of decreasing total lipid on heparin sensitivity ratio. Symbols as fig. 8.19.

TABLE 8.26 USFAR WITH INCREASING PS OR PI

PS					
mol %	3.6	7.2	10.8	14.5	18.1
ratio	0.84	0.80	0.75	0.71	0.67
PI					
mol %	3.2	6.5	9.8	13.2	16.6
ratio	0.87	0.85	0.84	0.82	0.8

Discussion - an important function of the APTT is its use in the laboratory monitoring of heparin therapy (Basu et al 1972). There is a good deal of variation in sensitivity to heparin among the commonly used commercial APTT methods (Poller et al 1980, Brandt and Triplett 1981).

The main aim of this experiment was to show whether alterations in relative concentrations of PS and PI influence sensitivity to the heparin-induced defect.

It is shown that the relative concentration of PS governs the sensitivity of the APTT test system to heparin. As the concentration of PS decreases senstivity to heparin increases. At the same time the procoagulant activity is reduced. Substitution of PS and PI was not beneficial; the procoagulant activity being markedly reduced and sensitivity to heparin remained unchanged over a wide range of concentration. In the experiment in which PS or PI concentrations were altered, the degree of unsaturation of the phospholipids was unimportant in promoting procoagulant activity and heparin sensitivity of the test system at the 100 mg dm^{-3} level of total lipid. As mentioned previously, Ploplis and Castellino (1980) and Tams et al (1979) considered that higher levels of unsaturation promote increased clotting activity. In expt 8.11, it was shown

that this effect occurred only when the total lipid concentration was below 100 μg dm^{-3}. The observed effects in this experiment are likely, therefore, to be due to changing in unsaturation.

It appears that in the APTT test system, lipid is in excess at levels over 30 mg dm^{-3}. Below this level lipid concentration becomes critical, with small reductions in concentration resulting in marked loss of procoagulant activity and an increase in heparin sensitivity. The loss of procoagulant activity on dilution of total lipid has been observed by Zwaal et al (1977). They showed a marked loss of procoagulant activity upon dilution of a phospholipid liposome which was designed to resemble the cytoplasmic surface of the platelet membrane.

Conclusions - heparin sensitivity may be adjusted by altering relative PS concentration. When total lipid concentration is less than 30 mg dm^{-3} it becomes critical for the clotting time and marked increases in sensitivity to heparin are obtained at extreme dilution.

The work described in this section formed the basis of a paper entitled "Lipid class composition and heparin sensitivity in the activated partial thromboplastin time" published in Thrombosis and Haemostasis Vol 50, pp 601-603 (1983), a copy of which is appended.

8.13 TO EVALUATE THE ROLE OF PS IN THE DIAGNOSIS OF LUPUS-LIKE ANTICOAGULANTS (LLA) BY THE APTT

Aim - to investigate further the mode of action of LLA and to determine which phospholipids are involved, with a view to simplification of laboratory diagnosis.

Procedure - for this experiment it was decided that prepared liposomes should be added to the Manchester APTT test system. This system already contained an aliquot of the Manchester APTT reagent, described in detail in experiments 8.1 to 8.6 above. The idea of addition of extra lipid follows from the observations of Triplett et al (1983) using lysed platelets.

Liposomes were prepared as described in Ch 6. The vehicle liposome was used in order to test a variety of phospholipids incorporated into it. PS, PI, PG, PA and lyso-PS were added in increasing concentrations from 10 mg dm^{-3} to 120 mg dm^{-3}. Liposomes containing PS were prepared with total lipid concentrations ranging from 100 mg dm^{-3} to 1600 mg dm^{-3}. The proportions of components remained constant. Preliminary tests showed that optimal effect in inhibiting the action of lupus-like anticoagulants was found using liposomes with a total lipid concentration of 400 mg dm^{-3} and this concentration was used in subsequent coagulation tests. For one experiment, the Manchester APTT was used to measure the clotting time of a plasma containing a LLA. The test was repeated using increasing concentrations of the reagent.

For coagulation studies, plasma samples from six consecutive patients with LLA (table 8.27) were compared with those from seven haemophiliacs with FVIII inhibitors. Plasma from patients with prolonged APTT due to a variety of other causes was also studied. The presence of a LLA was confirmed by demonstration of an immediate acting inhibitor, tissue thromboplastin inhibition and typical results in mixing with normal plasma with the kaolin clotting time. The APTT was measured by the routine method described in Ch 6. To this test system was added either 0.1 cm^3 of test liposome suspension or 0.1 cm^3 of buffered saline as control.

One stage assays of factors VIII and IX were performed on patients with lupus inhibitors using the Manchester APTT reagent as the source of lipid and repeated sequentially with the addition of the "standard liposome" suspension to both test and standard plasmas.

TABLE 8.27 CLINICAL DETAILS OF PATIENTS WITH LUPUS ANTICOAGULANT

Patient	Age	Sex	Primary Diagnosis	Venous Thrombosis	Recurrent Abortion	Arterial Occlusion
(a)	33	F	SLE		+	
(b)	23	M	SLE	+		+
(c)	25	F	SLE	+		+
(d)	26	F	Mixed Connective Tissue Disease	+	+	
(e)	78	F	*None			+
(f)	25	M	*None	+		

*no current evidence of connective tissue disorder

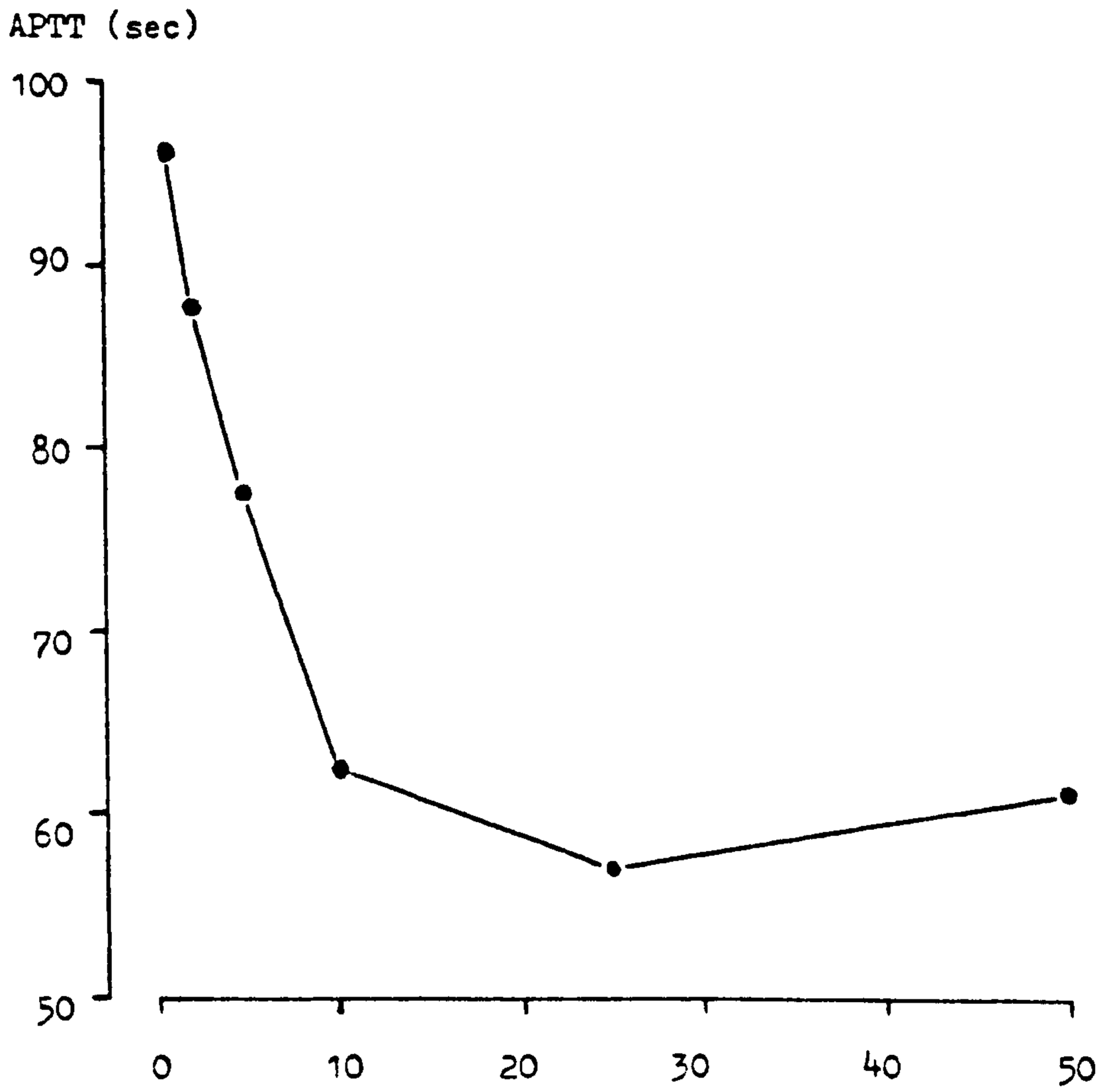


Fig.8.27 Effect of increasing concentration of APTT reagent on the APTT of a plasma containing a lupus anticoagulant.

TABLE 8.28 PLASMA CONTAINING A LUPUS ANTICOAGULANT
Effect of adding a standard four component liposome containing phosphatidyl serine.

Patient	APTT Seconds	Saline - APTT Seconds	PS liposome APTT Seconds	Difference APTT - PS Liposome APTT Seconds
Normal	42	45	39	3
(a)	80	73	56	24
(b)	96	101	49	47
(c)	108	100	55	53
(d)	65	64	54	11
(e)	109	99	57	52
(f)*	130	122	86	44
(g)	73	73	43	30

*same patient as (e) tested in different occasions when overdosed with oral anticoagulants (INR > 5.0).

TABLE 8.29 APTT OF PLASMA CONTAINING FVIII AND FIX INHIBITOR

Inhibitor		APTT sec.	Saline APTT sec.	PS-liposome sec.	Difference APTT - PS-liposome APTT
VIII	1.2 u/ml	265	272	264	1
VIII	1.48 u/ml	149	167	155	-6
IX	2.32 u/ml	245	239	265	-20
VIII	8.8 u/ml	174	207	189	-15
*VIII	10.6 u/ml	296	343	237	59
*VIII	9.2 u/ml	292	347	239	53
VIII	62 u/ml	172	212	213	-41
VIII	136 u/ml	171	213	218	-47

*same patient tested on different occasions.

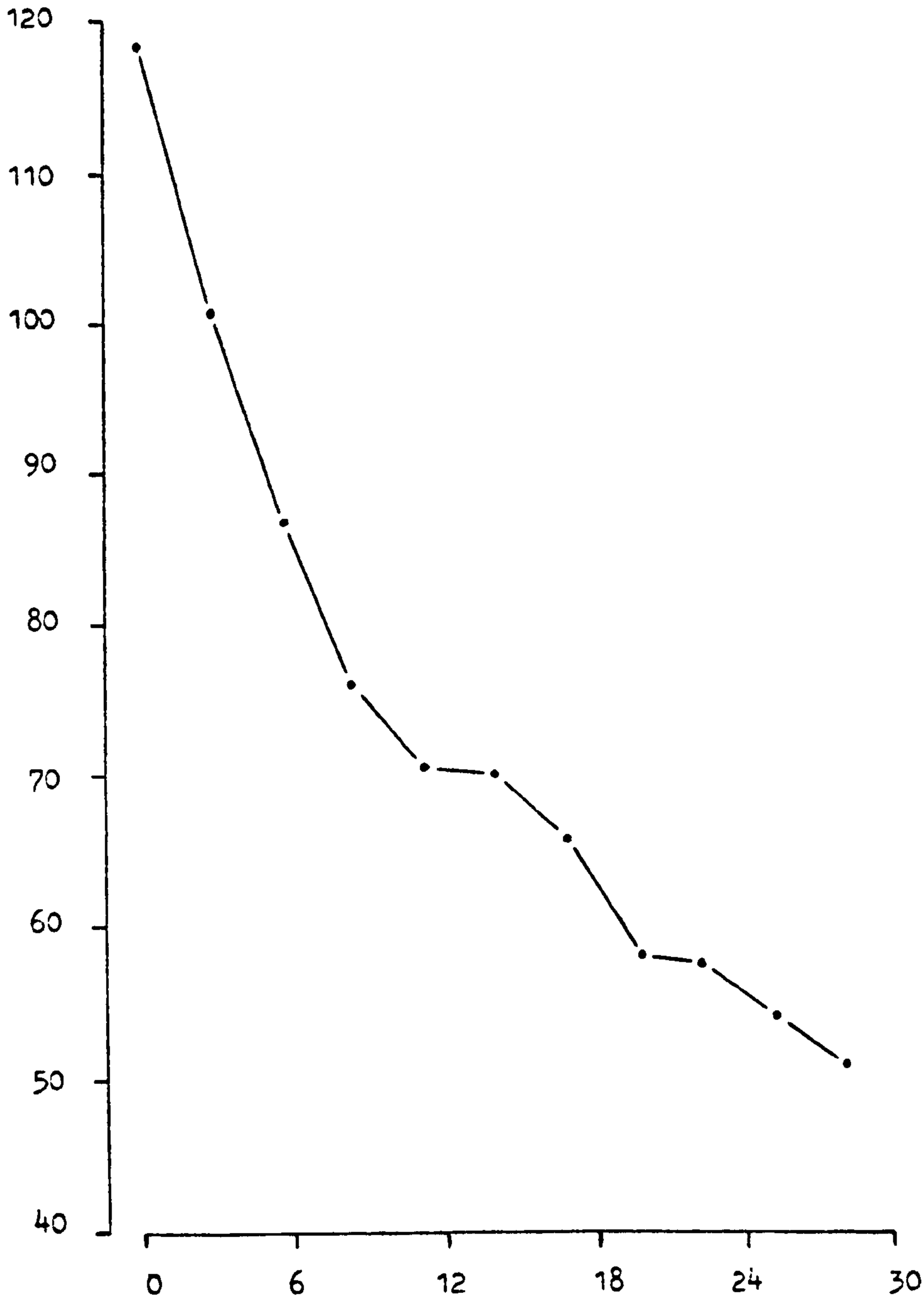


Fig. 8.28 effect on the APTT of a plasma containing a lupus inhibitor of addition of a liposome containing PS.

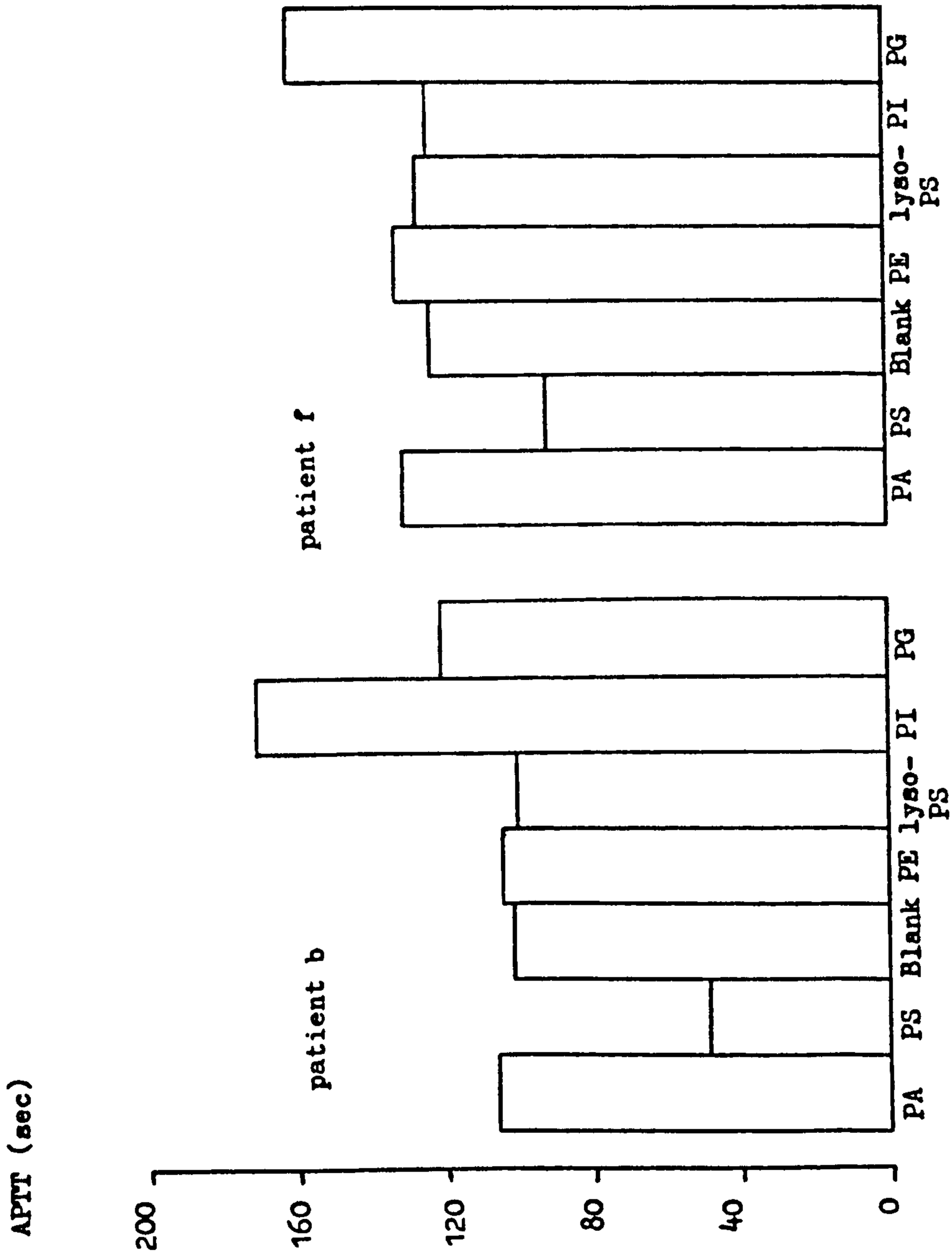


Fig 8.29 Effect on the APTT of plasmas containing a lupus anticoagulant of replacing PS with other phospholipids. Patients b and f as in Table 8.28.

TABLE 8.30 EFFECT OF ADDITION OF STANDARD PS-LIPOSOME TO PLASMA FROM PATIENTS WITH VARIOUS ABNORMALITIES

Clinical Situation	APTT seconds	Saline APTT seconds	PS-liposome APTT seconds	Difference APTT - PS-liposome seconds
Heparin	435	375	395	40
Heparin	84	92	65	19
Heparin + Coumarin	107	118	92	15
Coumarin	49	55	57	-8
Heparin + Protamin (Post Cardiac Bypass)	47	57	65	-18
Heparin + Protamine	45	53	55	-10
VIII Deficient(VIIIC=21%)	65	69	69	-4
VIII Deficient(VIIIC= 1%)	195	240	221	-26
IX Deficient (IX=30%)	63	74	73	-12
XI Deficient (XI=30%)	72	83	80	-8
XI Deficient (XI=24%)	52	58	68	-12
XII Deficient (XII=1%)	180	230	234	-54

TABLE 8.31 FACTOR IX ASSAY ON PATIENT WITH LUPUS-LIKE ANTICOAGULANT* Effects of Addition of Liposome Containing PS

Test Dilution	Factor IX without liposome	Factor IX with liposome
1/20	10%	40%
1/40	14%	40%
1/100	20%	40%
1/200	30%	40%

*patient on oral anticoagulants concomitantly

Results - an increase in the concentration of the Manchester APTT reagent corrected the prolongation of the test resulting from lupus anticoagulants (Fig 8.27). Similarly, the addition of a standard liposome containing 400 mg dm^{-3} of total lipid caused significant shortening of the APTT of all the lupus inhibitor plasmas tested (table 8.28). When the quantity of PS in a standard

liposome was varied (while maintaining the total lipid content at 400 mg dm^{-3}) there was progressive shortening of the APTT as PS content increased. This acceleration was significant as very small increases in PS were made (Fig 8.28).

When other phospholipids were substituted for PS in the same vehicle liposome and in the same proportion there was no shortening of the APTT (Fig 8.29). When plasma from patients with specific inhibitors to factor VIII and IX respectively were tested using the standard liposome, containing PS in the same proportion, there was only one patient in whom the APTT showed appreciable shortening. Even with this patient's plasma, however, the APTT was still excessively prolonged (> 200 sec) (Table 8.29).

Plasma from patients with a prolonged APTT due to factor deficiencies and anticoagulant therapy were tested in a similar manner. Significant shortening of the APTT occurred only in those patients receiving heparin. In patients with factor deficiencies the addition of the standard liposome lead to a prolongation of the APTT similar to that seen on addition of saline and which was ascribed to a dilution effect (table 8.30). When one stage assays of factor VIII and IX were carried out on the plasmas containing lupus inhibitors a characteristic pattern was observed in all cases. Non-parallel dilution response curves were found, with an apparent increase in the measured factor at high test dilution. In contrast, when the assay was repeated with the addition of the standard vehicle liposome containing PS, the dilution response curves became parallel indicating that the inhibitor had been

overcome. Table 8.31 shows an example of a factor IX assay on a patient with LLA who was receiving concomitant oral anticoagulation treatment. This was a typical pattern in all the lupus inhibitor plasmas tested.

Discussion - lupus-like anticoagulant is a spontaneously acquired immunoglobulin, usually lgG or lgM, that occurs in a variety of clinical situations and interferes with most phospholipid-dependent coagulation tests. Feinstein and Rapaport (1972) coined the term lupus anticoagulant twenty years after Conley and Hartmann first described two patients with systemic lupus erythematosis and a coagulation inhibitor. The disorder is not associated with a haemorrhagic diathesis unless some abnormality of clotting proteins or of the platelets is also present (Shapiro and Thiagarajan 1982). There appears to be an increase in thromobembolic events however, in patients with the abnormality, as well as a high rate of spontaneous abortion (Bowie et al 1963, Elias and Elder 1984, Carreras et al 1981, Ware Branch et al 1985). More recently, it has been shown that a high percentage of patients with AIDS possess the LLA (Bloom et al 1986, Cohen et al 1986).

The laboratory diagnosis of LLA is based on the presence of an immediate acting inhibitor, evidence of tissue thromboplastin inhibition and demonstration of the "lupus cofactor" effect i.e. the enhancement of the effect by the addition of normal plasma (Schleider et al 1976, Exner et al 1978). Recent work (Triplett et al 1983, Coots et al 1981) has shown that the tissue thromboplastin inhibition test is not sufficientlly specific and cannot reliably

distingish between inhibitors of the lupus type, specific factor inhibitors and other causes of prolonged APTT. Triplett et al (1983) have suggested using the addition of lysed platelets to the APTT system. It is confirmed that in an APTT test system the effect of the LA can be overcome simply by increasing the concentration of lipid available. Furthermore, PS plays a key role in governing this effect. The value of the vehicle liposome has again been demonstrated in providing a catalytic surface for coagulation factor interaction.

Antibodies to various phospholipids have been found in association with naturally occurring LLA (Thiagarajan and Shapiro 1983) and have also been demonstrated in monoclonal lupus autoantibodies produced by hybridoma techniques (Lafer et al 1981). It is widely assumed that the inhibitor acts by interference with the binding of vitamin K-dependent clotting factors to lipid surfaces and this effect has been demonstrated experimentally (Thiagarajan et al 1980).

In this experiment, firm evidence of the specificity of the effect of a PS-containing liposome is provided by the demonstration of acceleration of the prolonged APTT due to lupus-like anticoagulants and the lack of effect on prolonged APTT results caused by other types of inhibitor, factor deficiencies and oral anticoagulants.

Conclusions - it is further demonstrated that PS plays a unique role in APTT reagents. Also, support is given to the notion that

the LLA acts by competing with coagulation factors for sites on procoagulant lipid surfaces. Some evidence is given to explain the different sensitivities of various commercial APTT reagents to the LLA, (Manucci et al 1971, Brandt et al 1987), which may be due to differences in their total lipid content or in relative concentration of individual lipid classes. It is worthy of note that some commercial houses now produce APTT reagents said to be sensitive to the LLA when used in conjunction with their existing reagents to provide test systems of contrasting high and low lipid concentration e.g. Dade "Actin FSL", Biodata "Platelet Extract".

8.14 DISCUSSION

The activated partial thromboplastin time test is one of the most widely used tests in coagulation studies. Its main functions are to screen for intrinsic clotting factor defects and to monitor heparin therapy. In addition, the test may be used as a screening test for the detection of lupus-like anticoagulants. An ideal APTT reagent would be one which would show good sensitivity to all of these.

The considerable extent of the differences between the various APTT reagents' coagulation responses to a spectrum of disorders has been noted on many occasions (e.g. Poller and Thomson 1972, Sibley et al 1973, Hathaway et al 1979, Manucci 1982). Attempts have been made to improve the APTT test system by employing various activators, such as ellagic acid (Ratnoff and Crum 1964) or silica (Margolis 1961) and adjusting activation times and buffering of the test system.

The source of the reagent lipids, the relative concentration of constituent lipid classes and the physical form of the reagents has not been studied. The aim of the experimental series described in Chapter 8 was to examine some widely used APTT reagents to elucidate their morphology, coagulation responses and lipid composition and to determine whether any of these features correlated. There is no published evidence of similar approaches to this topic, on such a scale as presented in this thesis. It was intended that the information derived from these experiments could then be employed in

the task of defining the optimal lipid constituents required in test systems designed for specific functions, e.g. for the monitoring of heparin therapy and for the diagnosis of the lupus-like anticoagulant.

In experiment 8.1 it was shown that the five commonly-used APTT reagents examined possessed a common structure i.e. they were made up of liposomes. A great variety of size and form was observed. The measurement of size and surface charge was the subject of experiments 8.2 and 8.3 and again revealed wide variation between reagents.

An early attempt to relate structure to activity was made by Rouser et al (1958) and Rouser and Schloredt (1958) who concluded, after testing a series of natural and synthetic lipids, that there was a requirement for phosphatides to be in a dispersed colloidal particle in order to show activity. It was not until 1961 that Rouser et al described a technique to separate PE from PS. This method allowed Marcus et al (1962) to study PS in more detail in a clotting test system. It is unfortunate that Rouser's experiments were flawed, because of the contamination of PE with PS, but nevertheless he was correct in his conclusion about the "particulate" nature of the lipid. Wallach et al (1959) described injecting ethereal solutions of phosphatides into aqueous buffers to form suspensions with clot promoting activity - a technique now routinely employed to manufacture liposomes and known as reverse-phase evaporation (Sjoka and Papahadjopoulos 1980). The electron micrographs in Chapter 8.1 confirm the suggestion of Rouser

et al that a discrete structure is essential for lipids to optimally promote clotting. Chapter 8.3 dealt with the charge on the liposomes of the various APTT reagents. It was observed that all the reagent liposomes tested had a negative charge and that this feature showed negative correlation with APTT prolongation in FXI deficient plasmas and plasmas heparinised in vitro. In their experiments, Bangham (1961) and Papahadjopoulos et al (1962) used lipid vesicles in a clotting time test of normal plasma and found that activity in such a system correlated with negative charge. Table 8.19 shows that, in the present work, the only parameter that correlated with normal plasma APTT was the quantity of PS per test. The convenience of use of the Malvern Instruments Autosizer and Zetasizer devices was a great advantage in Chapter 8.2 and 8.3, allowing the speedy determination of size and electrophoretic mobility of reagent liposomes. The lipid composition of the five APTT reagents was determined in Chapter 8.4. Actin FS, made from soya beans, was shown to have a different lipid distribution from that of the other four reagents, of animal origin. These had a broadly similar distribution of phospholipid classes as shown in Fig 8.7, although there was a wide range of total lipid concentration per test (Table 8.6) with the Mancheser APTT containing 0.25 μg total phospholipid per test while Actin had 9.88 μg, Automated APTT had 12.28 μg and Activated Thrombofax 18.6 μg. Actin FS contained 63.31 μg. The reagents also showed differences in neutral lipid composition and in distribution of fatty acids. The ratios of unsaturated to saturated fatty acids (shown in table 8.8) ranged from 0.78 for Actin to 2.54 for Actin FS. Linoleic acid (18.2) was the major component of the unsaturated fatty acids of Actin FS. In

the reagents of animal origin the major unsaturated fatty acids were oleic (18:1), arachidonic (20:4) and docosahexaenoic (22:6). Stearic acid (18:0) was the major saturated fatty acid in these preparations.

Chapter 8.5 dealt with the performance of the five APTT reagents in testing the APTT of plasmas from a variety of normal and abnormal sources. Care was taken to test a sufficient number of plasmas for the derivation of a normal range for each reagent. It proved necessary to transform the normal data in order to achieve a Gaussian distribution. When this was done it was possible to calculate the ratios of the APTT of abnormal plasmas/higher limit of transformed normal range. Then, reagents were ranked according to their performance in measuring the APTT of the various abnormal plasmas. Fig 8.9 shows this ranking. A number of points of interest emerge from this experiment. The reagents showed varied sensitivities to a spectrum of coagulation disorders. This has been commented on previously, e.g., Sibley et al (1973), Manucci (1982), Thomson and Poller (1985). When the upper limit of the transformed normal range was used to calculate ratios, the major change observed was a lowering of the upper limit of normal in Actin, Automated APTT and Activated Thrombofax, with an increase in success rate in the detection of mild or border line coagulation defects. The implication is that great care must be taken in defining normal ranges. In chapter 8.6 an assessment was made of the foregoing experiments in order to test for correlation between the clotting performance data and the observations on size distribution, electrophoretic mobility and lipid composition. To do this, the

rank in clotting performance achieved by each of the reagents was compared with a number of observations of size, mobility and lipid composition using Spearman's Rank Correlation test. Table 8.19 shows those correlations that were significant at the 5% level.

Features that influence charge i.e. proportion and absolute concentration of PS and ratio of negatively charged phospholipid to total phospholipid are prominent in the table of significant correlations. These emphasize the importance of negative charge on the reagent liposomes.

The concentration of total phospholipid was said to govern the sensitivity of the method to the presence of lupus-like inhibitor (Manucci et al 1979). This was confirmed in the present study. Actin FS, with the highest concentration of phospholipids, ranked lowest with lupus-like inhibitor containing plasma while Manchester APTT, with the lowest phospholipid concentration, gave the highest ranking.

The next experiments were built on the base provided by those already discussed. By constructing liposomes of varying composition, it was hoped that the role of PS could be clarified, since it was clearly of the greatest importance. Table 8.19 showed that PS had influence in promoting potent clotting activity of normal plasmas and in sensitivity to FVIII and XI deficient plasmas, plasmas with a FVIII inhibitor or lupus-like inhibitor and sensitivity to the defect caused by addition of heparin to normal plasma in vitro.

It was decided that a liposome should be constructed of individual pure phospholipids and cholesterol. This liposome should be tested to show that there was no deterioration of its components and examined by electron microscopy to ensure that it was genuinely a liposome i.e. that it showed a lipid membrane enclosing an aqueous compartment. The liposome should then be tested in the APTT system by using it in place of the Manchester APTT reagent. Activity was expressed as % of that shown by batch 117 of the Manchester APTT reagent, the internal 'house' standard preparation in use at that time in the UK Reference Laboratory. It was hoped that this liposome would show no activity whatsoever in the APTT system. Chapter 8.7 shows that it was possible to prepare a liposome, whose structure was confirmed by electron microscopy, that possessed neither procoagulant nor anticoagulant activity in the APTT system. This liposome could serve as a vehicle for other phospholipids, therefore, while contributing no activity of its own to the test system.

We may conclude that, as Rouser suspected, there must be a particulate structure so that there can be activity in a lipid mixture, but also, that there can be similar structures without any activity whatsoever. The next experiment showed the effect on the activity in the APTT system of this vehicle liposome containing increasing amounts of PS. Procoagulant activity was conferred on the liposome by PS incorporation and was maximal at a molar concentration of PS of 1.8% and above. This was a most important observation and demonstrated that it was possible to construct a

liposome made from individual pure lipids which showed the same procoagulant activity as batch 117, extract of human brain lipids.

In the next experiment (Ch 8.9) a PS-containing liposome was tested in the APTT system over the range of concentration 100 mg to 10^{-5} mg dm^{-3} of total lipid. Fig 8.16 shows the loss of procoagulant activity upon dilution of the liposome. There is no evidence of any anticoagulant activity, contrary to reports by Marcus (1962), Troup and Reed (1958), Mustard et al (1962) and Turner and Silver (1963). These workers may have employed lipids that were not pure, in systems where liposomes were not formed.

In the next experiment (Ch 8.10), PS was replaced by a range of other phospholipids. Lyso-PS, PG and PI were used in place of PS in the vehicle liposome. A mixture of PI and PS was also employed instead of PS. The results showed that PS was unique in its ability to confer procoagulant activity on this liposome mixture. Lyso-PS and PI both showed low activity while PG was completely inert. This proved useful since it meant that PG could be used as a make-weight in later experiments. The activity shown by PI was of great interest since it could be expressed as procoagulant or anticoagulant depending on the circumstances. When PI was used in equimolar mixtures with PS, both these effects were greatly amplified.

In the next experiment the USFAR of the test liposomes was varied (see Ch 8.11). At high total lipid concentration there was little difference between the preparations with USFARs of 0.1, 0.54

and 0.67. The most saturated preparation, with USFAR of 0.04 had less procoagulant activity than the other mixtures at every concentration tested. This liposome showed strong anticoagulant activity upon dilution while the most unsaturated mixture showed procoagulant activity even at concentration of total lipid of 10^{-5} mg dm^{-3}. The degree of unsaturation of membrane fatty acids was shown to influence their clotting activity by Ploplis and Castellino (1980) and by Tans et al (1979). The experiment provides further evidence for this view and demonstrates that at total lipid concentrations below approximately 1 mg dm^{-3} a low USFAR liposome shows strong anticoagulant activity while a high USFAR preparation is procoagulant.

In Chapter 8.12 the role of PS in sensitivity to heparin is considered and it was shown that the relative concentration of PS in the vehicle liposome governed the sensitivity of the APTT test system to heparin. As the concentration of PS decreased the sensitivty to heparin increased. Substitution of PS by PI was not beneficial. It appeared in these experiments that total lipid was in excess at concentrations above 30 mg dm^{-3}. Below this concentration, lipid becomes the limiting factor, with small reductions in concentration resulting in marked loss of procoagulant activity and an increase in heparin sensitivity. Zwaal et al (1977) observed the loss of procoagulant activity that followed dilution of total lipid in a liposome designed to resemble the cytoplasmic side of the platelet membrane.

The final experiments in the series are described in Chapter 8.13 and were designed to evaluate the role of PS in the diagnosis of lupus-like anticoagulants by the APTT test. Manucci et al (1979) and Brandt et al (1987) have observed the different sensitivities of various APTT reagents to the LLA. Further evidence of this difference is presented above and a possible explanation is presented in the light of the experiments in Chapter 8.13 of the role of PS in detection of the presence of a LLA.

These experiments support the view that the LLA acts by competing with coagulation factors for binding sites on procoagulant lipid surfaces. A reagent which is insensitive to the presence of the LLA is likely to have a high concentration of total lipid and be rich in PS while a sensitive reagent will show the reverse i.e. low total lipid concentration with relatively low PS. The use of both sensitive and insensitive reagents may offer a better diagnostic test. This observation has been acted upon by some commercial producers and a number of alternative lipid preparations are now available for this test e.g. the Platelet Extract Reagent made by the BioData Corporation of Hatboro, USA, and Actin FSL made by Dade.

In Chapters 2 and 4, there was some discussion about the form of membranes and of lipids in hydrated systems. In preparing the liposomes described above a standard technique was employed i.e. lipids were dissolved in solvents, usually chloroform or chloroform: methanol mixtures, taken to dryness under oxygen-free nitrogen and then hydrated. The aqueous phase used in this step was heated, before addition to the dried lipid, to a temperature above the

highest transition temperature (Tc) in the lipid mixture and sonicated at this temperature. The fact that these preparations formed liposomes, on the one hand, as was demonstrated in Fig 8.11, and performed well in the APTT, on the other, shows that a membrane was available for the interaction of the clotting proteins that bore a reasonable resemblance to a natural membrane. A number of workers have reported on the way in which hydrated lipids form liposomes. Berden et al (1975) and Barsukov et al (1980), using nuclear magnetic resonance, observed that vesicles composed of PC with acidic phospholipids formed with the latter predominantly located in the inner monolayer. In vesicles of PC and PS the latter was located predominantly on the inner side at low pH but preferred the outer side at higher pH. At given pH values and sizes of vesicles, the final transmembrane distribution depended upon the relative area occupied by the polar head groups of each phospholipid and on their surface charges. Cullis and de Kruyff (1979) examined lipid polymorphism in membranes and were of the opinion that the shape of a particular phospholipid molecule was of the greatest importance in regulating what sort of phase it would form, e.g., lyso-phospholipids and detergents were shown to be in the shape of an inverted cone with the polar head at the non-pointed end. These molecules spontaneously form micellar or $hexagonal_{II}$ phase. PE, and cardiolipin, and phosphatidic acid in the presence of Ca^{2+}, form a $hexagonal_{II}$ phase, as do PA at pH < 3 and PS at pH < 4.0. In the case of these molecules, the polar head group is at the pointed end of the cone. PC, SPH, PS, PI, PG, PA and cardiolipin form cylindrical shapes and, according to these authors, readily form lamellar phase. There is great disagreement, however, about

the way in which membranes form in hydrated systems. Several reports in favour of the spontaneous formation idea have been published (Israelachvili et al 1975, 1976, 1977, 1980 and Mitchell and Ninham 1981). An alternative view was presented by Helfrich (1974), Fromherz (1983), and Lasic (1982) who picture the phospholipid vesicle as a distortion of a planar membrane which requires energy for its formation and which is inherently unstable. Cornell et al (1982) showed that the minimum radius obtained for liposomes was independent of the hydrocarbon chain length when this was in the range from 12 to 18 carbons. The minimum radius of egg yolk PC vesicles was 10.7 $\pm$ 0.3 nm. This result was opposite to the findings of Israelachvili (1977). Cornell et al (1986) concluded that the spontaneous vesicle formation model only applies to a limited class of molecules where the electrostatic term (a term which in PC, for example, describes the interaction energy between the phosphorus atom of the phosphate group and the nitrogen atom of the choline group) is the dominant free energy contribution which drives the bilayer to a more curved geometry. In the case of PC they suggest that the flexibility of the zwitterion eliminates this term causing the planar geometry to be the preferred state. The phase behaviour of complex mixtures of acidic and neutral polar lipids tends to be dominated by the charged component. Gounaris et al (1983) made detailed studies of polar lipid extracts from chloroplast thylakoid membranes dispersed in aqueous systems. They observed that small bilayer vesicles were formed in distilled water. When the charge on the major acidic lipids was neutralized by reducing the pH or by addition of low concentrations (< 5 mmol dm^{-3}) of metal cations, fusion of the vesicles resulted and a

phase separation of the non-bilayer forming lipids followed with formation of inverted micelles sandwiched within a lipid bilayer. De Kruiyff et al (1979) and Verkleij et al (1979) studied the effect of polyvalent ions on cardiolipiin in mixed layers of cardiolipin and PC. They showed that a complex of Ca^{2+} with cardiolipin formed hexagonal$_{II}$ phase very readily. Stabilising the lipid phase therefore appears to be an important role for charged lipids. Cullis and Verkleij (1979) showed that in the absence of Ca^{2+}, PS stabilizes the bilayer structure formed by an extract of red blood cell and platelet lipids.

A recent study by Rauch et al (1986) showed that hexagonal$_{II}$ phase phospholipids, including natural and synthetic forms of PE were able to neutralize the lupus anticoagulant activity of hybridoma antibodies. Lamellar phospholipids such as PC and synthetic lamellar forms of PE had no effect on these antibodies. The experiments described in Chapter 8 on the subject of the lupus anticoagulant showed a specific effect of a PS-containing liposome on the APTT of plasma containing the lupus-like inhibitor. In these experiments it appeared that the shortening of the APTT was not due simply to absorption of antibody to excess antigen, but to provision of extra phospholipid surfaces in the test system to which both clotting factors and the inhibitor may bind competitively.

In conclusion, the experiments described in this chapter show the nature of some commonly used APTT reagents and their lipid composition. The clotting performance of the reagents was then assessed and correlated with lipid compositional data. The

information derived from this work enabled the construction of a "vehicle" liposome which possessed neither procoagulant nor anticoagulant activity in the APTT test. Electron microscopy demonstrated that a liposome was formed. The introduction of PS into the vehicle rendered it active in the clotting test. PS was unique and shown to be of central importance in regulating procoagulant activity, sensitivity to heparin and to the presence of the lupus-like anticoagulant.

It is hoped that this work may provide a base for preparation of improved APTT reagents or possibly Reference Preparations, constructed, perhaps, from individual pure lipids.

CHAPTER 9 EXPERIMENTS ON FREEZE-DRYING

The six experimental series in this chapter have been designed to demonstrate the freeze-drying conditions required for tissue thromboplastin reagents. In the first section, residual moisture was determined in a series of reagents freeze-dried in a standardised manner. This freeze-drying sequence is described. The next two sections consider the use of liquid nitrogen cooling in the freeze-drying machine and its effect on the process. In the fourth section, an examination is made of the freeze-drying machinery to uncover possible sources of variation in residual moisture levels that may be due to position of vials within the plant or to machine design. Section 5 describes the preparation of tissue thromboplastin reagents intended to serve as international reference preparations for the Bureau Communautaire de Reference of the European Economic Community, the World Health Organisation and the International Committee for Standards in Haematology. Finally, the stability of thromboplastin reagents in fusion-sealed glass and rubber-stoppered vials is compared.

9.1 TO DETERMINE THE RESIDUAL MOISTURE IN TISSUE THROMBOPLASTIN REAGENTS FREEZE-DRIED UNDER STANDARDISED CONDITIONS

Aim - To establish a value for residual moisture (RM) in freeze-dried tissue thromboplastin reagents.

Procedure - Nineteen batches of human brain thromboplastin were lyophilised over a period of eight years between March 1976 and November 1984. These were batches which were free of gross bacterial contamination and hepatitis B virus (Microbiological testing is described in Chapter 6.18). Thromboplastin was dispensed as described in Chapter 6.12. Fourteen batches were aliquoted into 2 cm^3 glass vials, shown in fig 6.17. Two batches were dispensed into glass vampoules illustrated in the same figure. A further batch was dispensed into polypropylene vials and two others into 10 cm^3 glass vials. In every case the procedure was performed using the Comp-u-pet apparatus (fig 6.23). For batches 80/120 and 82/253, a special dispensing head was made for vampoules. This is also shown in fig 6.23. After dispensing, vials were partially stoppered using stopper type A in fig 6.18 and vampoules using stopper type B. The material was then cooled to -50^{o}C in a mechanically cooled refrigerator (Kelvinator UC 517, Laboratory Impex, Teddington). The mean cooling rate was 2.5^{o}C per hour. Freeze-drying was as described in Chapter 6.12 and followed the sequence illustrated in fig 9.1, with a condenser temperature of -65^{o}C. When drying was complete, the vials were stoppered in vacuo and the material transferred to a refrigerator operating at -20^{o}C for storage. Residual moisture was measured as described in Chapter 6.12.

Results - Table 9.1 shows the batch number, the type of enclosure used, the preparation date and the residual moisture of the test preparations. The mean value for residual moisture was 1.84% with a standard deviation of 0.18. Fig 9.1 shows the freeze-drying sequence employed for the lyophilisation of tissue thromboplastin. The entire process took five days; one day for cooling of load and machine, approximately three days of sublimation, and one day of desorption over P_2O_5. Fig 9.1 shows the process, beginning with the cooling of the freeze-drier shelves (dotted line) and the thromboplastin (solid line). After the thromboplastin was loaded and the vacuum pump started there was an immediate reduction in pressure, measured at head 1 (i.e. near to the vacuum pump - see Chapter 6). The drop in pressure at head 2 was not as marked because of the subliming water vapour from the load. This vapour was trapped on the condensing surface and caused it to rise in temperature. Meanwhile, the load and shelf temperatures equilibrated, and it was then necessary to switch on the shelf heating to provide the energy to support sublimation. As the process continued, head 2 pressure dropped to equal that shown by head 1. This indicated that the sublimation stage of the process was complete.

The load temperature at this time was $10^{o}C$, confirming that there could be no residual ice in the thromboplastin and that further prolongation of the sublimation phase was pointless. Accordingly, the desorbtion stage was begun. The rate of heating was increased and during this stage desorbing vapour was entrapped

in the P_2O_5 trap, while the mechanically cooled condenser was defrosted. The process was completed when the load temperature reached 20°C and no further drop in pressure was noted.

TABLE 9.1 DETAILS OF LYOPHILISED TISSUE THROMBOPLASTIN

Batch No.	Enclosure type	Date	Residual moisture (%)
76/005	1	Mar 1976	1.5
77/007	1	Jun 1977	2.1
77/025	1	Sep 1977	2.1
77/047	1	Dec 1977	1.7
78/048	1	Mar 1978	1.5
78/074	1	Aug 1978	1.7
79/090	1	Mar 1979	1.9
79/091	1	Mar 1979	2.0
*79/099	1	Jul 1979	1.9
79/106	1	Oct 1979	2.1
80/120	2	Mar 1980	1.8
81/178	1	Dec 1981	2.0
82/235	1	Jul 1982	1.7
**82/253	2	Jul 1982	1.9
83/283	1	Feb 1983	1.7
83/286	1	Mar 1983	1.9
83/313	3	Jun 1983	1.7
84/382	4	Apr 1984	1.9
***84/441	4	Nov 1984	1.8

* Reference Preparation of the EEC Bureau de Communautaire de Reference

** International Reference Preparation of the World Health Organistaion

*** Reference Preparation of the International Committee for Standards in Haematology

Key to vial type
1 = 2 cm^3 glass vial, 13 mm stopper
2 = 1 cm^3 glass vampoule, flame sealed
3 = 4 cm^3 polypropylene vial, 13 mm stopper
4 = 10 cm^3 glass vial, 20 mm stopper

Discussion - The nineteen batches of thromboplastin reagent

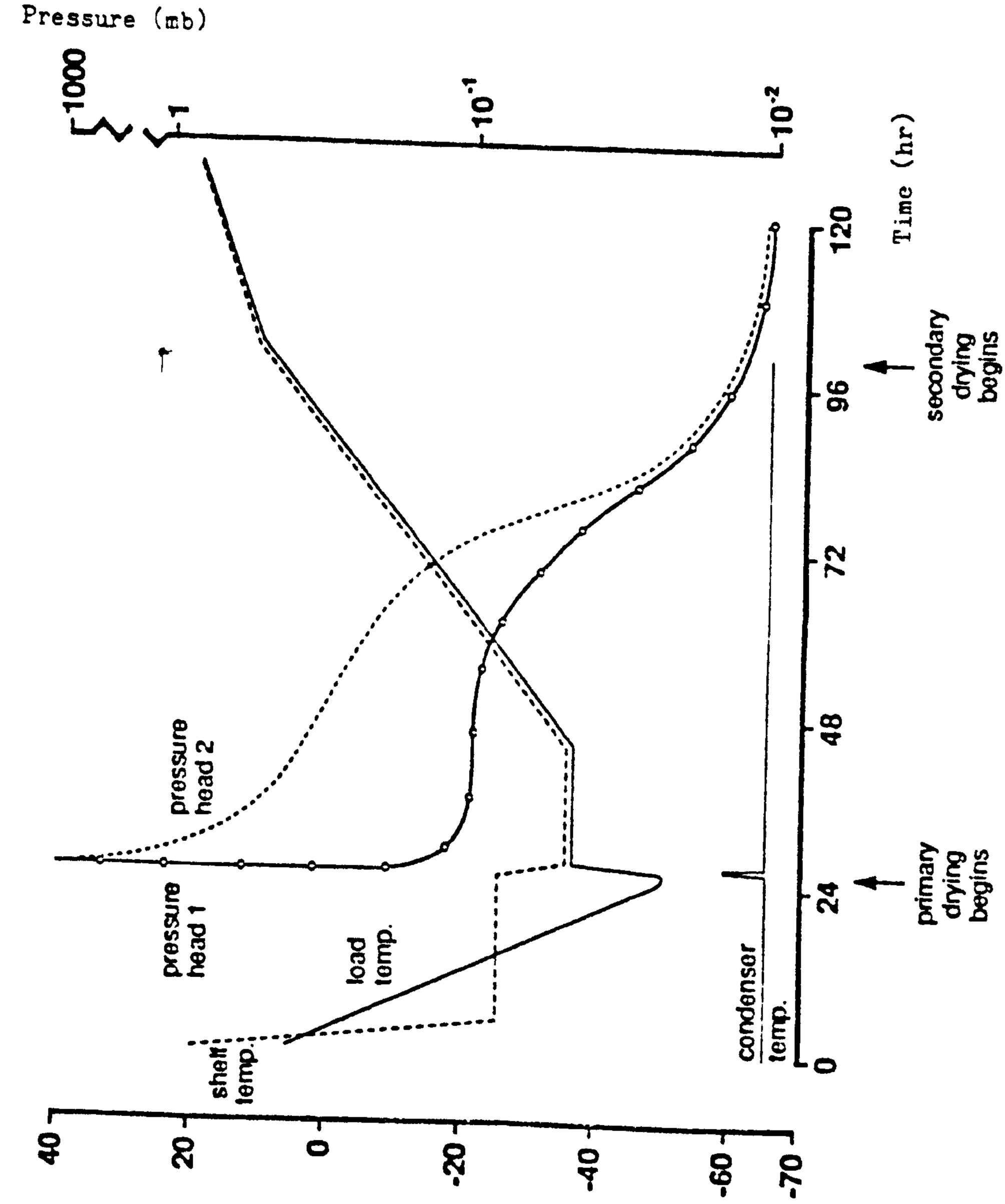


Fig 9.1 Freeze-drying sequence for tissue thromboplastin.

lyophilised between 1976 and 1984 were prepared in the same way and freeze-dried according to the sequence described. Batch 82/253 was prepared for the World Health Organisation and therefore had to be presented in a fusion sealed glass enclosure. For this reason the reagent was dispensed into vampoules, rather than the standard freeze-drying vial (see Chapter 6.12). Batch 80/120 was treated in the same way and acted as a practice run for the WHO material so that the dispensing, freeze-drying and flame sealing equipment could be tested thoroughly. The other types of vial employed were a 4 cm^3 capacity polypropylene vial with 13 mm neck, and an approximately 10 cm^3 capacity glass vial with a 22 mm neck (see fig 6.17). While there are not enough data to compare the effect of vial geometry on residual moisture, it may be seen that the enclosures labelled 2, 3 and 4 gave results that fell within the range shown by vial type 1. The standard deviation of the residual moisture values is encouragingly low and indicates that the treatment of the material was reasonably standardised. The freeze-drying sequence produced thrombplastins with an acceptable level of residual moisture for long-term storage. For example, BCT 76/005, prepared in March 1976, was used as a house standard at the UK Reference Laboratory until supplies were exhausted in June 1979, i.e. more than 3 years of use. BCT 78/074, prepared in August 1978, was used until late 1982. BCT 79/099, prepared in July 1979 is currently a Reference Preparation of the EEC Bureau Communtaire de Reference, more than ten years after its manufacture. The stability of this preparation has been the subject of an independent investigation by van den Besselaar et al (1988) who found no evidence of any change in the material.

Conclusion - The mean residual moisture value for a range of thromboplastin reagents, freeze-dried in a variety of vials, was found to be 1.84%. This value appears to be adequate for long-term stability of the reagents. The freeze-drying sequence employed produced consistent results over the time of the experiment.

9.2 OBSERVATIONS ON THE USE OF A LIQUID NITROGEN-COOLED CONDENSER IN THE FREEZE-DRYING MACHINE

Aim - To observe the effect of employing very low condenser temperatures during freeze-drying of tissue thromboplastin.

Method - For this experiment, the mechanically-cooled condenser was removed, and replaced by the device shown in fig 6.14. This involved disconnection of the mechanical system and its controls, and the installation of the liquid nitrogen-cooled condenser. The system required a supply of liquid nitrogen, provided by a self-pressurising dewar (BOC Cryospeed EC Series, BOC Cryospeed, Worsley). This was connected to the condenser by a nylon pipeline of 1/2 inch outside diameter, under the control of a thermocouple activated valve and controller. Temperature could be set from ambient down to -196°C. For safety, the liquid nitrogen pipelines were insulated and fitted with "over-pressure" valves which were designed to open if the pipeline was closed at both ends causing pressure to build up within it. Exhaust liquid nitrogen was piped to the outside of the building.

A batch of thromboplastin reagent was prepared, dispensed into 2 cm^3 glass vials and frozen as described above. It was then divided into three and each third freeze-dried in a separate freeze-drying run with condenser temperature set at -80°C, -120°C and -160°C respectively. Following drying, the vials were sealed in vacuo and residual moisture measured as described in Chapter 6.12, on ten groups of five vials from each run. Liquid

nitrogen usage was calculated for each run by referring to the capacitance liquid nitrogen level gauge fitted to the EC Series dewar. Analysis of variance and Tukey test were employed to analyse the data.

Results - Fig 9.2 shows the conditions that obtained in the three freeze-drying runs. Reducing the condenser temperature resulted in a quicker drop in pressure. The warming effect of subliming vapour on the condensing surface temperature was less marked as condensing temperature was lowered. The ultimate vacuum achieved was not changed by lowering condenser temperature. Table 9.2 lists the residual moisture values for the three thromboplastin batches. Table 9.3 shows results of analysis of variance which indicated a significant interaction between residual moisture and condenser temperature. The Tukey test was used to test for difference between the means of the residual moisture values for each temperature. There was strong evidence (at the 0.1% level) to show that drying with the condenser temperature at $-80^{o}C$ removed less moisture than at the other two, lower, temperatures. There was evidence, at the 5% level only, to show that drying with the condenser temperature at $-160^{o}C$ removed more moisture than at $-120^{o}C$.

Fig 9.3 reveals that a reduction in residual moisture is coincident with reduction in the temperature of the condensing surface. The value for residual moisture at a condenser temperature of $-65^{o}C$ was that calculated in Chapter 9.1.

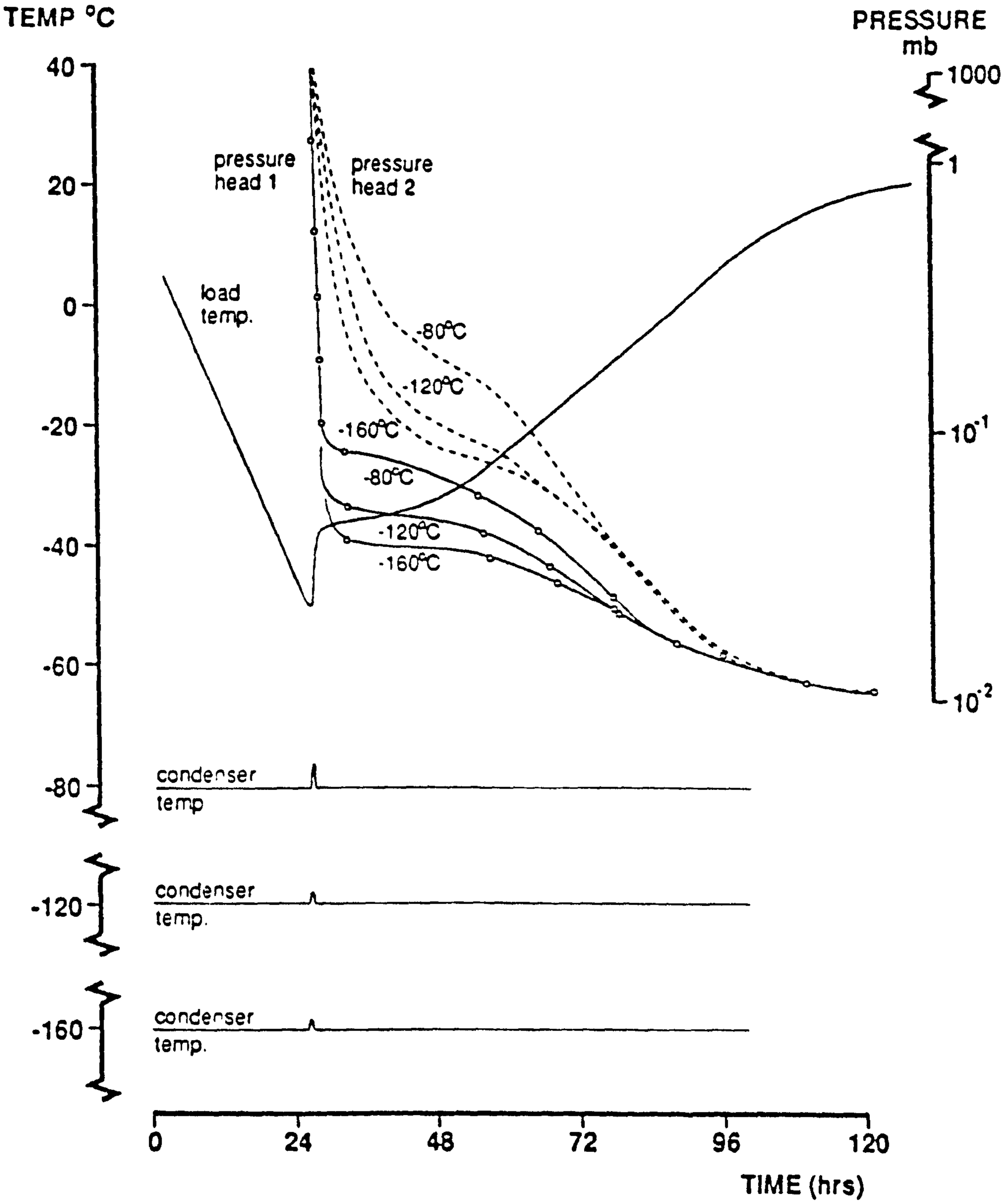


Fig 9.2 Freeze-drying sequences with very low condenser temperatures.

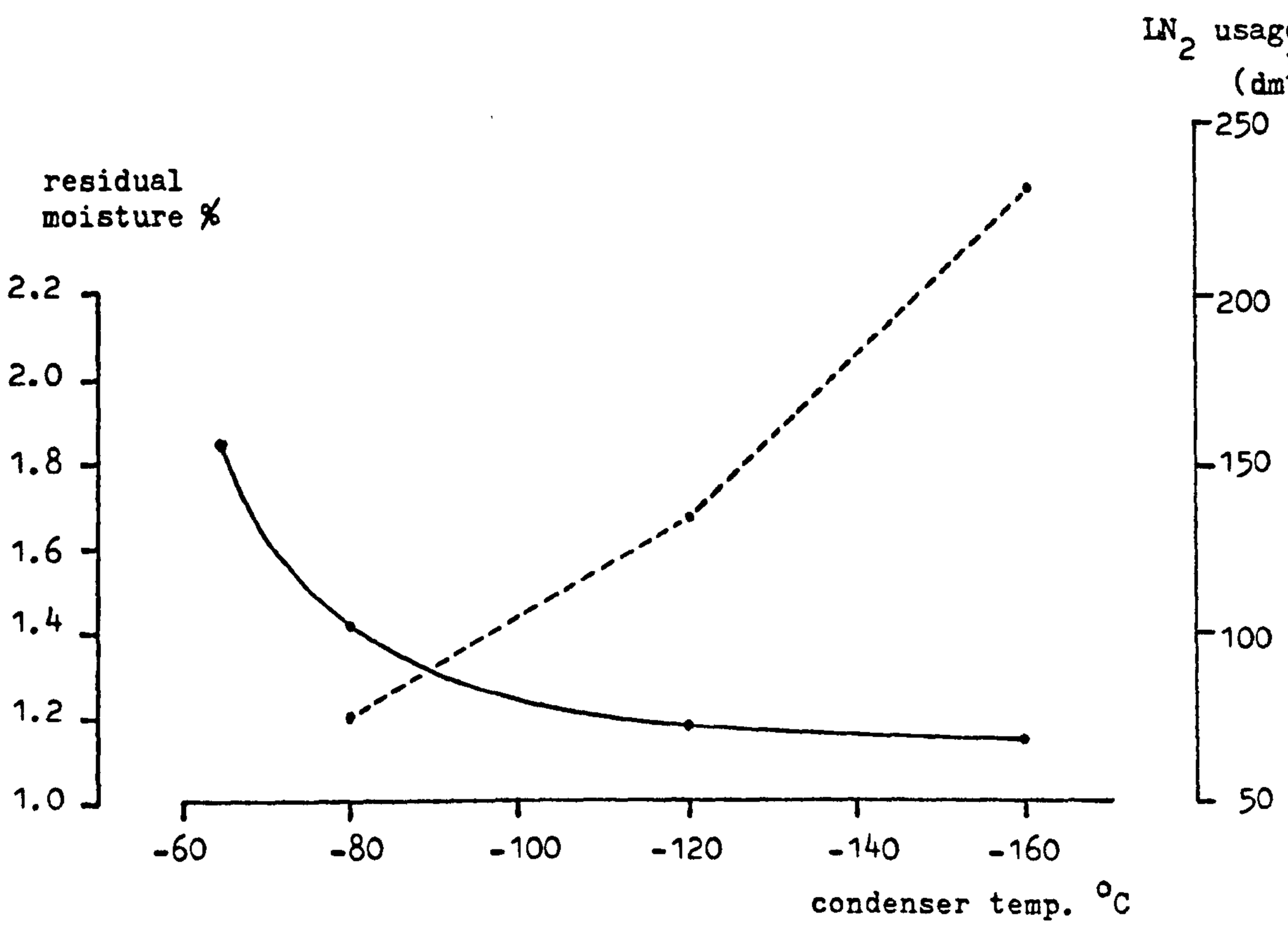


Fig 9.3 Relationship between condenser temperature and residual moisture. Usage of liquid nitrogen (LN_2) is also shown.

TABLE 9.2 PER CENT RESIDUAL MOISTURE (RM) IN LYOPHILISED TISSUE THROMBOPLASTIN

Condenser temperature		-80°C	-120°C	-160°C
RM estimate	1	1.4	1.16	1.16
	2	1.38	1.17	1.14
	3	1.38	1.19	1.14
	4	1.37	1.2	1.16
	5	1.38	1.17	1.14
	6	1.4	1.16	1.14
	7	1.4	1.16	1.14
	8	1.38	1.19	1.15
	9	1.39	1.17	1.13
	10	1.39	1.18	1.15
mean		1.39	1.18	1.15
standard deviation		0.01	0.01	0.01

TABLE 9.3 ANOVA OF THE DATA IN TABLE 9.2

Source	df	SS	MS	F	p
temperature	2	0.3480	0.174	1269.73*	<0.0001
residual moisture	27	0.0037	0.000137		
total	29	0.3517			

Discussion - It is clear that the use of the liquid nitrogen-cooled condenser had a marked effect on the freeze-drying process. Theoretical discussions on the subject of the sublimation of ice have been offered by Carman (1948) and more recently by Mellor (1978). They proposed that for effective sublimation, the total pressure within the system must be less than the vapour pressure of the material at the evaporating surface. Also, the vapour pressure at the condensing surface must be lower than the vapour pressure of ice at the surface of evaporation. As discussed

in Chapter 6, the vapour pressure at a condenser surface will be at minimum if the accommodation coefficient is unity. Kramers and Stemerding (1951) calculated that the probability of a molecule of water sticking to a condensing surface at -60°C or lower was unity. Since the condenser temperature in the three freeze-drying cycles described here was well below this level, this must be the case. The result is that the vapour pressure at the condensing surface reached a low level very quickly and that this improved the efficiency of the freeze-drying. Lower levels of residual moisture coincided with lower condenser temperature.

The data show, however, that the reduction in residual moisture was very small when condenser temperature was lowered from -120°C to -160°C and that this reduction required some 95 dm^3 of liquid nitrogen. This use of liquid nitrogen is difficult to justify, given that in Chapter 9.1 it was shown that a residual moisture level of 1.84% was perfectly adequate for long-term storage of tissue thromboplastin.

A further point of interest concerns the design of the condensing surface. Rowe (1964b) considered that the area of the condensing surface should be at least equal to the shelf area in a freeze-dryer. The liquid nitrogen-cooled condenser employed in this experiment had a surface area of 0.74 m^2, or 19.07% of shelf area. The mechanically-cooled condenser used in Chapter 9.1 had a surface area of 1.11 m^2, or 28.6% of shelf area. It is clear that both produced acceptable results, indicating that design and

operating temperature are more important considerations than surface area per se.

Conclusion - It was possible to dry to lower residual moisture levels by employing very low temperature condensing surfaces. The cost of liquid nitrogen for cooling, however, (approximately 20p per dm^3) may be prohibitive, and small reductions in residual moisture values may not justify the additional expense.

9.3 OBSERVATIONS ON THE USE OF A LIQUID NITROGEN-COOLED TRAP TO REPLACE PHOSPHORUS PENTOXIDE DURING THE DESORPTION PHASE OF FREEZE-DRYING

Aim - To observe the effect of replacing the phosphorus pentoxide trap with a device cooled by liquid nitrogen.

Method - The stainless steel trays used to hold P_2O_5 were removed from the freeze-drying plant and the device shown in figure 6.16 fitted in their place. This apparatus comprised a tubular reservoir, to hold liquid nitrogen, to which was attached a series of fins, made of brass, to present the maximum cooled surface. The entire device was nickel plated to prevent corrosion and to ensure a smooth surface for easy cleaning. Liquid nitrogen was pumped into the reservoir, via a thermostatically controlled solenoid valve, from a BOC EC 200 self-pressurising dewar. The temperature was set to $-120^{o}C$. Exhaust nitrogen vapour was piped to the outside of the building.

A batch of human brain thromboplastin was prepared and divided into two. The first was lyophilised using the liquid nitrogen cooled trap during the desorption stage while the second used P_2O_5 to entrap desorbed vapour. The sequence of freeze-drying shown in figure 9.1 was followed. Residual moisture was measured in samples from each batch. Data was analysed using Students t test. Liquid nitrogen use was measured using the capacitance gauge fitted to the EC 200 dewar.

Results - Residual moisture values for the two freeze-dried batches are given in Table 9.4. The batch dried using the liquid nitrogen-cooled trap had a lower residual moisture level, confirmed by the t test ($p < 0.001$). Thirty-one dm^3 of liquid nitrogen was used.

TABLE 9.4 RESIDUAL MOISTURE IN FREEZE-DRIED THROMBOPLASTIN

	Liquid nitrogen trap	P_2O_5 trap
1	1.74	1.90
2	1.77	1.92
3	1.77	1.90
4	1.76	1.86
5	1.78	1.89
6	1.77	1.88
7	1.74	1.87
8	1.76	1.90
9	1.75	1.91
10	1.78	1.89
mean	1.76	1.89
standard deviation	0.015	0.015

Discussion - Desorption using the liquid nitrogen cooled apparatus gave a preparation with a lower residual moisture. This may have been due to the improved entrapment of desorbing vapour by the device, leading to a lower vapour pressure within the freeze-drying plant. This, in turn, would have favoured a more efficient drying. Freeze-drying by this method did not result in the very low residual moisture levels seen in Chapter 9.2 when a liquid nitrogen-cooled condenser was employed, but the quantity of liquid nitrogen used, i.e. some 30 dm^3, meant a more economical means of achieving lower residual moisture was possible. Defrosting

and cleaning of the device was simple, requiring only that it be washed with hot water and a major advantage was that the use of P_2O_5 was obviated.

Conclusion - A liquid nitrogen trap, used in place of P_2O_5, had several advantages. Firstly, lower residual moisture was obtained by its use; secondly, it proved more economical of liquid nitrogen than the condenser used in Chapter 9.2; thirdly, it was easy to defrost and clean and; finally, it avoided the use of P_2O_5 and the dangers associated with this compound.

9.4 TO TEST FOR INTRINSIC MACHINE VARIATION IN A BATCH OF FREEZE-DRIED TISSUE THROMBOPLASTIN

Aim - To discover whether there were any measurable variations in a batch of tissue thromboplastin freeze-dried in the plant described above.

Method - In order to discover whether there were any significant variations within materials lyophilised in the plant described it was necessary to test four possible areas of potential variation. 1. variation from front to back on a shelf, 2. variation from edge to middle of a shelf, 3. variation from top to bottom of the shelf array within one chamber, 4. variation from chamber to chamber.

Accordingly, a batch of human brain thromboplastin was prepared as described above. The material was dispensed in 1.5 ml aliquots into the polypropylene vial shown in fig 6.17. In all, some 3200 aliquots were dispensed. This number provided sufficient for the immediate study and enough for a reasonable batch to present for external monitoring for routine use.

The vials were arranged as follows. Six freeze-drying trays, each holding 25 rows of 20 vials were placed in chamber no. 1 of the freeze-drying plant. Chambers 2, 3 and 4 were filled with freeze-drying trays packed with vials of water and 5 vials per tray of thromboplastin. This represented the maximum load that the machine was designed to accommodate, i.e. 18 dm^3.

The freeze-dryer was then run as shown in fig 9.1 above, and the vials stoppered in vacuo at the completion of the process.

To test for variation from front to back, the six trays of dried thromboplastin were removed from chamber 1 and 10 vials were removed from rows 1, 7, 13, 19 and 25 from tray 1. These groups of 10 vials were further divided into two lots of 5 so that two residual moisture determinations could be made per row (i.e. 10 per tray). This sampling was repeated on each tray from chamber 1.

To test for variation from edge to middle, a batch of 12 vials was removed from the middle of each tray from chamber 1. These were the 9th, 10th, 11th and 12th vials from rows 12, 13 and 14. "Middle" vials were compared with material sampled from the outermost columns of vials on each tray i.e. 4 groups of 5 vials from each edge column, to provide eight values per tray.

To test for variation from top to bottom of the shelf array in chamber 1 the data already generated was used and front of shelf values compared with edge of shelf and middle of shelf values. The mean value of all the data per shelf was calculated for comparison.

To test for chamber to chamber variation, the mean values from chamber 1 were compared wtih those from chambers 2, 3, and 4.

Results - The results of residual moisture testing of samples from chamber 1 are given in table 9.5.

TABLE 9.5 RESIDUAL MOISTURE IN SAMPLES FROM CHAMBER 1

Row No	1	7	13	19	25
Shelf no 6	1.81 1.81	1.81 1.8	1.8 1.81	1.81 1.81	1.82 1.82
5	1.81 1.81	1.81 1.81	1.8 1.81	1.82 1.8	1.8 1.8
4	1.81 1.8	1.82 1.81	1.81 1.8	1.82 1.81	1.8 1.79
3	1.82 1.82	1.82 1.8	1.82 1.81	1.78 1.8	1.81 1.79
2	1.81 1.82	1.81 1.81	1.79 1.8	1.8 1.8	1.79 1.8
1	1.81 1.8	1.82 1.82	1.81 1.81	1.82 1.8	1.8 1.81

Analysis of variance applied to these data give the results shown in table 9.6.

TABLE 9.6 ANOVA OF DATA IN TABLE 9.5

Source	df	SS	MS	F	p
shelf	5	0.0003333	6.666×10^{-5}	1.22	0.34
row	4	0.0007067	1.75575×10^{-4}	3.23*	0.03
column	1	0.0001067	1.067×10^{-4}	1.95	0.18
shelf x row	20	0.002433	1.2165×10^{-4}	2.22*	0.04
shelf x column	5	0.0003733	7.466×10^{-5}	1.36	0.28
row x column	4	0.0001267	3.1675×10^{-5}	0.58	0.68
residual	20	0.0010933	5.4665×10^{-5}		
total	59	0.0051733			

The analysis of variance show that there is no evidence to indicate difference between shelves, columns or any column interactions with rows or shelves. There is evidence at the 5% level to show a difference in rows and a shelf x row interaction - i.e. the rows do not return the same result in exactly the same way on each shelf. A plot of the row mean values is given in fig 9.4 and is very confusing because of the large number of shelves and rows. Interaction between shelves and rows is indicated by lack of parallelism. Tukey's range test was applied to the row means, and

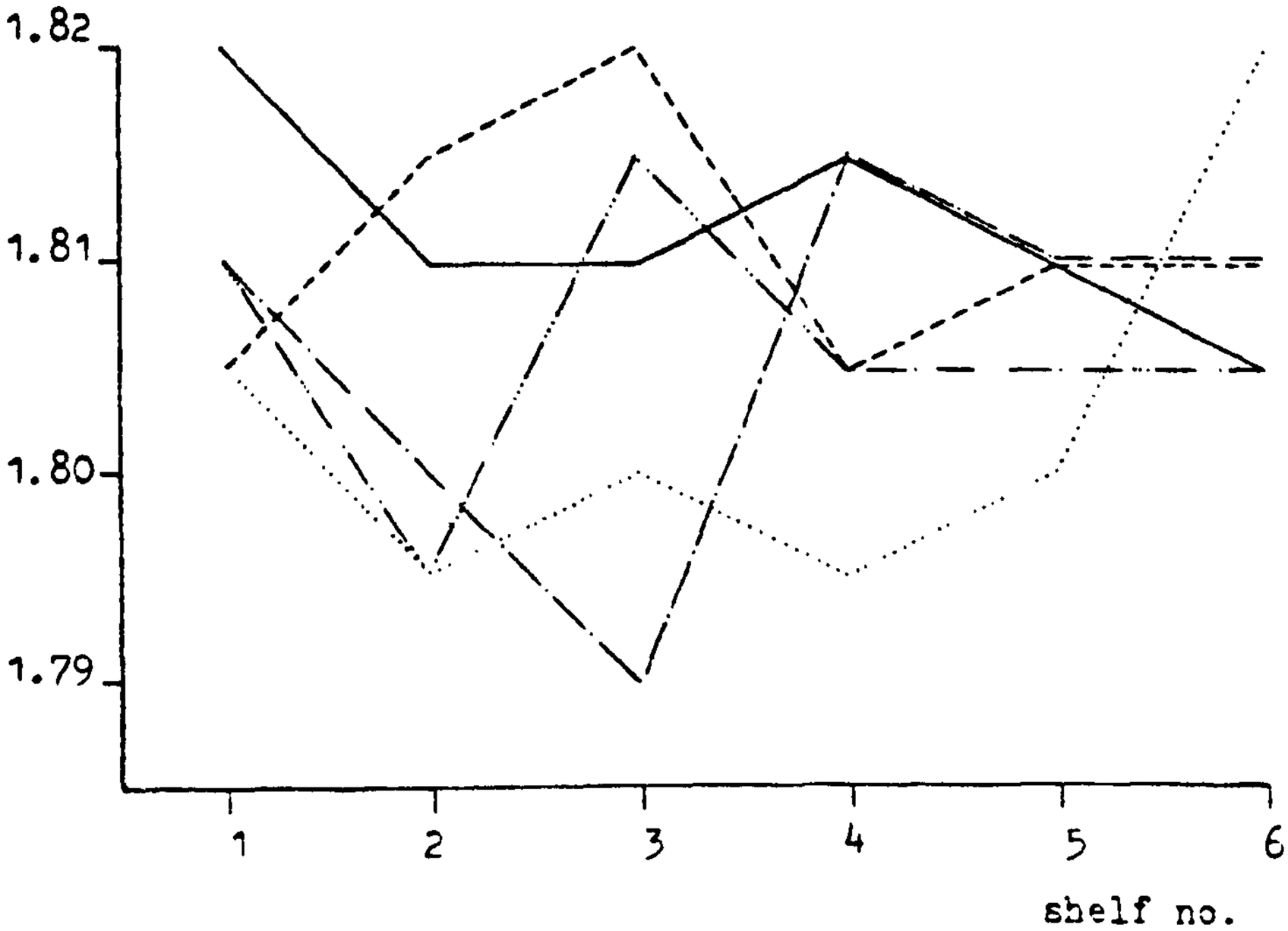


Fig 9.4 Lack of parallelism in lines joining row means for each shelf.

showed that the critical distance parameter for the data was 0.009. This meant that if a difference between rows was to be significant, at the 5% level, it must be greater than 0.009. This was the case for the difference between rows 2 and 7 (0.01, $p < 0.05$) but was not the case for the next largest difference i.e. rows one and 25 (0.009, $p = 0.05$). The only significant finding, therefore, was that row 7 had a higher residual moisture than the others.

The residual moisture values of materials sampled from front row, back row and left and right edge columns i.e. 12 samples per tray are shown in table 9.7. The middle vial residual moisture results are also given in this table.

Analysis of variance failed to show any difference between positions, or between shelves, as shown in table 9.8. To achieve significance, the F value would have to have been 2.71 or more for shelves and 2.87 or more for positions.

All the data generated so far were used, in an analysis of variance to show if any differences could be found.

There was no evidence to show a difference in residual moisture for different rows or columns or any interactions. There was evidence (at 1% level) that there was a difference between shelves ($F = 4.83$ in table 9.9). Tukey's test was employed to show whether the difference between shelf residual moisture means was significant. The means of the shelves in chamber 1 are shown in table 9.10.

TABLE 9.7 RESIDUAL MOISTURE FROM EDGES AND MIDDLE OF FREEZE-DRYING SHELVES

Shelf	Front row		Left column		Back row		Right column				Middle
6	1.81	1.81	1.80	1.80	1.79	1.80	1.82	1.82	1.81	1.80	1.80	1.83	1.81
5	1.81	1.81	1.80	1.79	1.81	1.80	1.80	1.80	1.80	1.79	1.80	1.81	1.81
4	1.81	1.80	1.79	1.79	1.80	1.81	1.80	1.79	1.80	1.81	1.79	1.80	1.81
3	1.82	1.82	1.83	1.82	1.81	1.79	1.81	1.79	1.80	1.82	1.80	1.81	1.81
2	1.81	1.82	1.81	1.82	1.81	1.81	1.79	1.80	1.81	1.82	1.80	1.80	1.80
1	1.81	1.80	1.82	1.82	1.81	1.81	1.80	1.81	1.82	1.83	1.81	1.81	1.81

TABLE 9.8 ANOVA OF DATA FROM TABLE 9.7

Source	df	SS	MS	F	p
shelf	5	0.0004635	9.27×10^{-5}	2.20	0.09
position	4	0.0002133	5.3325×10^{-5}	1.27	0.31
residual	20	0.00084167	4.20835×10^{-5}		
total	29	0.0015185			

TABLE 9.9 ANOVA (ALL DATA)

Source	df	SS	MS	F	p
shelf	5	0.001835	3.67×10^{-4}	4.83*	0.0081
row	3	0.0002896	9.653×10^{-5}	1.27	0.32
column	1	0.00001875	1.875×10^{-5}	0.25	0.63
shelf x row	15	0.001723	1.149×10^{-4}	1.51	0.22
shelf x column	5	0.0004188	8.376×10^{-5}	1.10	0.40
row x column	3	0.0002729	9.097×10^{-5}	1.20	0.34
residual	15	0.0011396	7.5973×10^{-5}		
total	47	0.0056979			

TABLE 9.10 SHELF MEAN RESIDUAL MOISTURE VALUES

Shelf no	Residual moisture
1	1.82
2	1.81
3	1.81
4	1.80
5	1.80
6	1.80

The Tukey test showed that there was no significant difference between shelves. Tukey's range test was also applied to the shelf mean data. In this test the "critical distance" statistic was found to be 0.022. Since the largest difference between shelves was 0.017 this means that there was not a significant difference between shelves ($p > 0.05$).

Table 9.11 shows an analysis of variance applied to mean residual moisture data from shelves and from chambers.

TABLE 9.11 ANOVA (MEAN RESIDUAL MOISTURE, SHELVES AND CHAMBERS)

Source	df	SS	MS	F	p
chamber	3	0.00085	2.833×10^{-4}	9.17**	0.0014
shelf	5	0.0005878	1.756×10^{-4}	3.80*	0.02
residual	15	0.0004635	3.09×10^{-5}		
total	23	0.0019013			

The ANOVA shows that there is evidence at the 1% level to show a difference between chambers and, at the 5% level, between

shelves. Shelf means (over all chambers) and chamber means (overall shelves) may be seen in table 9.12.

TABLE 9.12 SHELF AND CHAMBER MEANS

	Shelf	Chamber
1	1.82	1.81
2	1.81	1.82
3	1.81	1.82
4	1.81	1.80
5	1.81	
6	1.82	

Application of the Tukey test to the data shows that there was no significant difference between chamber 1 and chambers 2 and 3 (which were identical) or between chamber 1 and chamber 4. The difference between chamber 4 and chambers 2 and 3 was significant, at the 5% level. Chambers 1, 2, and 3 did not differ significantly.

Shelves 4 and 5 were the same and gave the lowest values. The largest difference was between 6 and 4 and 5 and was significant (at 5% level). The next largest difference was between 2 and 6 but was not significant. Differences between shelves 1, 2, 3 and 6 were not significant.

The overall mean residual moisture value was 1.81% with a standard deviation of 0.01. The highest recorded value was 1.83% and the lowest was 1.78%. The coefficient of variation of the residual moisture measurements was 0.51%.

Discussion - The mean value for residual moisture was found to be 1.81% with a standard deviation of 0.01 and CV of 0.51%. This shows a much narrower range and CV than might have been expected. The method of choice - the "heat and weigh" method, described in Chapter 6.12 gave a standard deviation of 0.06 and a CV of 3.04% for a sample of twenty determinations with a mean residual moisture of 2.1%. The 114 determinations used in the current experiment have given rise to an improvement in standard deviation and CV. The statistical methods employed may have been over sensitive in showing the significant differences reported, but nevertheless, the overall performance of the freeze-drying machine and thromboplastin lyophilisation sequence were encouraging.

The "heat and weigh" method has been advocated by a number of authors e.g. Seligmann and Farber (1971), Pemberton (1977) and May et al (1982), and their comments and suggestions were borne in mind when the apparatus described in chapter 6.12 was designed. May et al (1989) reported wide variations in the CV calculated for a range of lyophilised biological materials, from 46% for a batch of immune globulin down to 1.9% for measles vaccine. They found that CVs were smaller for samples having a residual moisture over 1%. Large CVs were found in samples with residual moisture less than 1%.

When the freeze-drying plant was designed, a decision was made to utilise four chambers, each containing six shelves with an area of 0.16 m^2. Commercial scale freeze-drying machines are built with only one or two shelves in a chamber, each of relatively enormous surface area. The evidence from this experiment is that

the small shelves offer good conditions for freeze-drying to low residual moisture and consistent inter-vial variation.

Conclusion - The data show a narrow range overall and all are within the coefficient of variation of the method. Some differences were statistically significant but do not upset the general picture of a system free of gross variation.

9.5 PREPARATION OF TISSUE THROMBOPLASTIN REAGENTS TO SERVE AS REFERENCE PREPARATIONS FOR THE EEC, WHO AND ICSH

Aim - To prepare extracts of tissue thromboplastin from human brains, to freeze-dry these reagents and present them in a form acceptable to the EEC, WHO and ICSH for use as international reference preparations.

Methods - Thromboplastin was extracted from human brains as described by Stevenson (1978). For the EEC batch, designated 79/099, aliquots of 1.5 cm^3 were dispensed into the vial type 1 in fig 6.17. For the WHO batch (82/253) aliquots of 0.75 cm^3 were dispensed into the vampoule shown in the same figure. This was used so that the reagent could be sealed in glass following drying. The ICSH reagent, labelled 84/441, was dispensed in 1.5 cm^3 aliquots into the vial type 4 in fig 6.17.

The Comp-u-pet apparatus described in Chapter 6.12 and pictured in fig 6.23 was used for dispensing the reagents. In the case of 82/253, the Comp-u-pet was fitted with a special dispensing head, shown in fig 6.23, designed for filling vampoules. The weight of material dispensed was tested for inter-vial variation by dispensing every hundredth aliquot into a pre-weighed vial and calculating the dispensed weight by difference. The coefficient of variation was calculated from this data. The reagents were lyophilised according to the sequence shown in fig 9.1. Following drying the vampoules, containing batch 82/253 for WHO, were flame sealed as described in Chapter 6.12. Residual moisture determinations were carried out, as

in this same section, and inter-vial variation in prothrombin time testing was assessed by using the reconstituted reagent in the measurement of the prothrombin time of a single plasma. The minimum acceptable number of vials included was 28 (Stevenson 1978). To determine the optimal concentration of phenol in their reconstitution fluids, sample vials from each reagent were reconstituted with a graded series of phenol solutions and tested for procoagulant activity and sensitivity to the coumarin-induced clotting defect. Each reagent was calibrated in an international multicentre exercise to ensure its suitability as a reference preparation, and a series of degradation studies was set up to monitor stability of the materials. Calibration exercises have been described elsewhere (Thomson et al 1984, 1986, Hermans 1984, Kirkwood 1984, Tomenson 1984) as have the proposed degradation studies on the WHO preparation 82/253 (Thomson et al 1984). Long-term degradation studies on the other two international reference preparations, 79/099 and 84/441, have been on-going since their manufacture. On each occasion of testing, at approximately three month intervals, the reagent was used to measure the prothrombin time of three test plasmas. The test plasmas were pools of donations from coumarin-treated patients or normal volunteers. The pooled plasmas were aliquoted into screw-topped plastic containers (Starstedt Ltd, Leicester) and stored in liquid nitrogen. The plasma pools have been replaced at approximately 3 year intervals.

Vacuum testing of vials was performed as described in Chapter 6.12 and microbiological tests were carried out as described in Chapter 6.18.

Results - Table 9.13 shows the test results for the three reagents described.

TABLE 9.13 CHARACTERISTICS OF THREE INTERNATIONAL REFERENCE THROMBOPLASTIN REAGENTS

	79/099	82/253	84/441
CV of dispensed weight of reagent	1.77%	1.69%	1.79%
Residual moisture	1.9%	1.9%	1.8%
CV of prothrombin time of a single plasma tested using 28 vials of reagent (mean PT in brackets)	1.6% (16.1 secs)	1.5% (16.9 secs)	1.6% (13.5 secs)
Phenol concentration in reconstitution fluid mmole dm^{-3}	5.3	2.7	18.6
International Sensitivity Index	1.048	1.085	1.04
Vacuum	tested by high-frequency spark tester		
Hepatitis B surface antigen	negative	negative	negative
HIV competitive ELISA	negative	negative	negative

The results of the long term degradation studies on batches 79/099 and 84/441 are given in fig 9.5.

Discussion - All three thromboplastin reference preparations described are still in use at the present time. The oldest, 79/099, was prepared in July 1979 and shows no evidence of instability after more than 10 years of use as a reference preparation of the EEC. Van den Besselaar et al (1988) confirmed this observation. The stability of this reagent may have surprised those proponents of the idea that sealing in glass is essential for long term storage of biological standards. (In the next section, butyl rubber stoppered vials are compared with fusion-sealed glass). The WHO stipulates that their reference preparations must be sealed in glass. Batch 82/253 was prepared in this way in order to comply with this regulation. It, too, is unchanged since its preparation (Thomson 1990 unpublished observations) and stability studies are still ongoing. Some results of degradation studies of batch 82/253 at elevated temperature are given in Chapter 9.6. The most recent reagent described, batch 84/441, was freeze-dried in rubber -stoppered vials also. It has proven to be stable to date.

As noted above, drying to a residual moisture level of less than 2% may be important for long term stability. Taking care over filling weight of reagents and inter-vial variation also contributed to the final character of the reagents and enabled them to fulfil the role of international reference preparations. Multicentre calibrations indicated good sensitivity (i.e. low ISI values) in all three reagents.

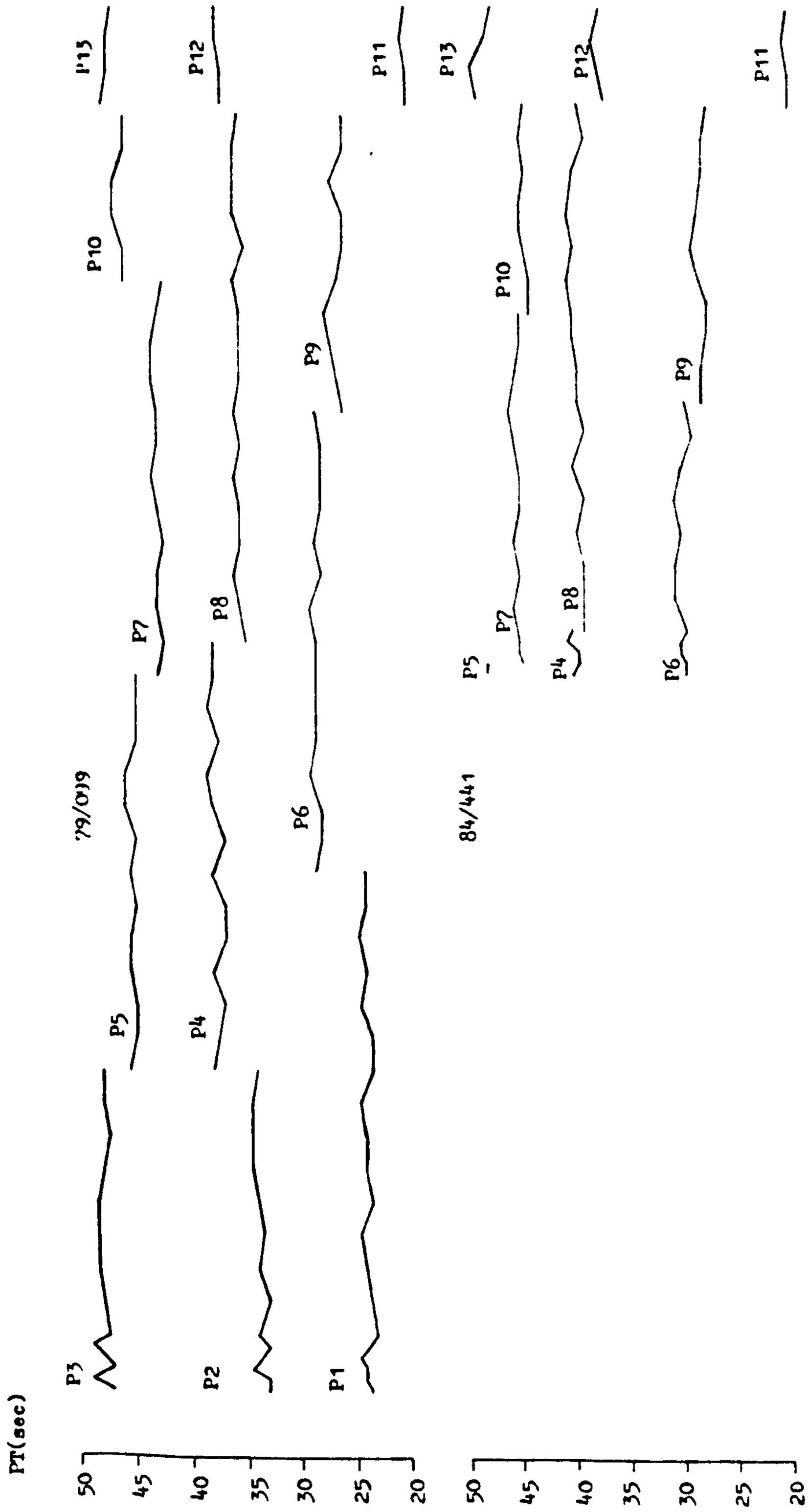


Fig. 9.5 Stability studies of batches 79/099 and 84/441

Conclusion - The most desirable quality of a reference preparation is that it should be stable over many years. It should also be sensitive to the coumarin induced defect (i.e. have an ISI close to unity) and show minimal inter-vial variation. Freedom from potential hazards to users is a further desirable feature. All these requirements have been met by the reagents described in this experiment.

9.6 TO EXAMINE THE STABILITY OF HUMAN BRAIN THROMBOPLASTIN FREEZE-DRIED IN VIALS WITH BUTYL RUBBER STOPPERS AND IN FUSION-SEALED VAMPOULES

Aim - To show whether material stored in fusion-sealed vampoules has a longer shelf-life than that stored in butyl rubber-stoppered vials.

Methods - Two batches of human brain thromboplastin were employed for this experiment, 80/120 and 82/253. Batch 80/120 was prepared to test the vampoule filling apparatus and the vampoule sealing device described in Chapter 6.12 and to act as a trial run for freeze-drying thromboplastin in vampoules, before proceeding to batch 82/253, the WHO reference preparation described in Chapter 9.5. The major part of 80/120 was dispensed into vampoules and approximately 200 cm^3 into vials. 82/253, on the other hand, was dispensed entirely into vampoules. Both batches were frozen and lyophilised according to the cycle shown in fig 9.1. When the process was complete all the vampoules containing 80/120 were fusion sealed. The majority of vampoules of 82/253 were also fusion sealed, as described in Chapter 6.12, but approximately 200 were stored with their butyl rubber stoppers still in place. Therefore, both batches included fusion-sealed vampoules and rubber stoppered vials or vampoules. These were vacuum tested before storage at $-20^{o}C$ or at $37^{o}C$ for heat-accelerated degradation studies.

Batch 80/120 was tested for stability by using the reconstituted reagent to test the prothrombin time of two plasmas, N1, a normal

control, and P2, from an anticoagulated patient. The plasmas were stored in liquid nitrogen. Batch 80/120, in vampoules, was tested at monthly intervals for six months, then three monthly. Batch 80/120 in rubber stoppered vials, was tested weekly. Batch 82/253 was tested by using the reconstituted reagent to measure the prothrombin time of two plasmas, N2, a lyophilised normal plasma, and P4, a plasma obtained from an anticoagulated patient. 82/253, in fusion-sealed vampoules, was tested at weekly intervals for 4 weeks, 3 months followed by 3-monthly intervals while the material in rubber-stoppered vampoules was tested weekly. At each time of testing, samples were also removed from the -20°C store and tested in the same way.

Vacuum testing was performed on rubber-stoppered vials and vampoules at weekly intervals and six monthly on fusion-sealed vampoules using the spark tester as described in Chapter 6.12. A disadvantage of this device was its inability to show a discharge glow when testing vials with a pressure of >100 mb. It was decided, therefore, to employ the method according to Barbaree and Smith (1981), also described in Chapter 6.12, to demonstrate whether there was still "negative" pressure in the rubber stoppered vials and vampoules which had failed the vacuum test by the spark tester method.

Residual moisture was determined also as described in Chapter 6.12, at baseline, two weeks and four weeks in the case of rubber-stoppered vials and vampoules, and 6 monthly in the case of

fusion-sealed vampoules. Malondialdehyde was determined, at the same intervals, by the method described in Chapter 6.20.

Results - The accelerated degradation study on 80/120 is shown in fig 9.6. The line drawn at 36.3 sec gives the baseline prothrombin time value + 10% for the anticoagulated plasma. This value was chosen to represent the maximum limit below which the thromboplastin could be regarded as maintaining stability. The line at 21.2 sec is the equivalent baseline + 10% limit for plasma N1.

The stability of 80/120 in fusion-sealed vampoules at 37°C was some 25 months with the coumarin plasma, P2, and 32 months with the normal plasma, N1. The material in rubber-stoppered vials, however, showed poor stability, exceeding the acceptable limit with both test plasmas by two weeks. Results obtained from material stored at -20°C are shown by the dotted line. These are seen to be stable over the period of the experiment.

The study of 82/253 showed a similar pattern (see table 9.14) with the rubber-stoppered material going outside the set limit by three weeks whle the fusion-sealed preparation took some 27 months for plasma N2 and 30 months for P4. Results obtained using material stored at -20°C are also given and indicate stability after five years.

The fusion-sealed vampoules in every case retained vacuum and the materials lyophilised in these containers showed no change in residual moisture or in mda content. See table 9.15.

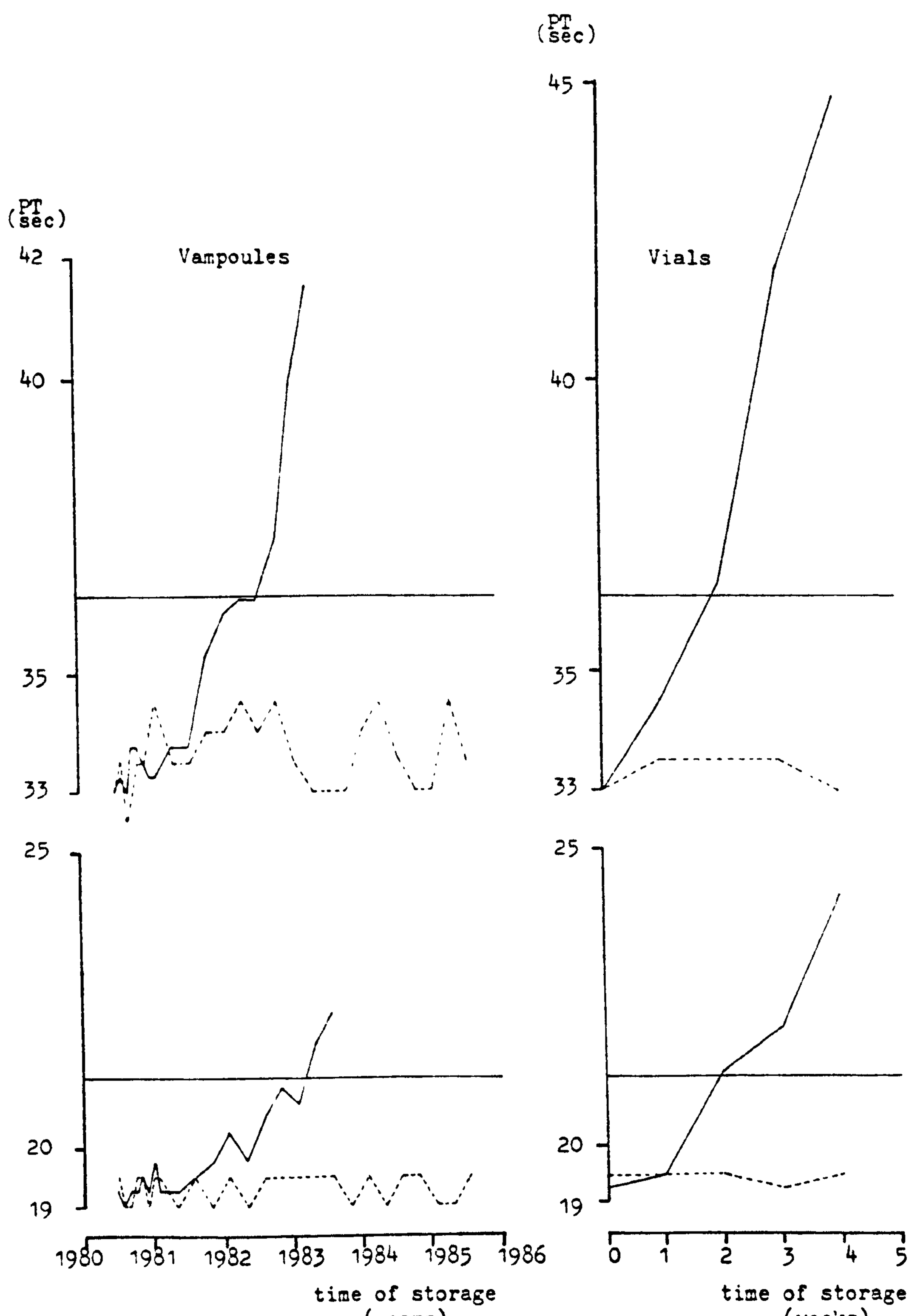


Fig. 9.6 Degradation study of 80/120 in vampoules and vials. Solid line shows material stored at 37°C while the broken line shows that stored at -20°C. The horizontal solid line is the baseline +10% value for each test plasma. Below this line the thromboplastin is regarded as having maintained stability.

TABLE 9.14 HEAT ACCELERATED DEGRADATION STUDY OF 82/253, PT(sec) OF NORMAL (N_2) AND COUMARIN (P_4) CONTROL PLASMA

	rubber-stoppered vampoules at 37°c.		fusion-sealed vampoules at 37°c.		fusion-sealed vampoules at -20°c.	
	N2	P4	N2	P4	N2	P4
baseline	17.7	36.2	17.7	36.2	17.7	36.2
week 1	17.5	38.5	17.5	37	17.5	37.5
week 2	17.5	39.5	17	36.5	17.5	37
week 3	20.5	41.5	17	37	17.5	37.5
week 4	23	42	17.5	37	17.5	37
3 months			17	36	17.5	37
6 months			17.75	37	18	36.5
9 months			17.5	37.5	18	36
12 months			18	37	18.5	36.5
15 months			18	37.5	18.5	36
18 months			19	38	18	37
21 months			18.75	38.5	17.5	36.5
24 months			19	39.5	17.5	37
27 months			19.5	39.5	18	37.5
30 months			19.75	40.5	17.5	37
36 months					17.5	37
42 months					18	37.5
48 months					17.5	37
54 months					17.5	37.5
60 months					18	37.5

N2 baseline + 10% = 19.47 secs. P4 baseline +10% = 39.82 secs.

TABLE 9.15 RESIDUAL MOISTURE AND MALONDIALDEHYDE IN 80/120 AND 82/253 IN FUSION SEALED VAMPOULES, AT 37°C

	residual moisture %		mda (nmole/ml)	
	80/120	82/253	80/120	82/253
baseline	1.83	1.90	11	17.5
12 months	1.86	1.90	12	18.5
24 months	1.88	1.88	10.5	18.5
30 months	1.85	1.91	11.5	18.0

There were no spark test failures in the vials of 80/120 stoppered with the type A stopper in fig 6.18. The combination of stopper type B and vampoules was less successful, however, and a failure rate of 6% was noted by 4 weeks. Testing by the method of Barbaree and Smith showed that there was still negative pressure in the vampoules, i.e., pressure between atmospheric and approximately 100 mb. The observation that there was sufficient vacuum to cause the syringe piston to close partially in this test implies that the pressure must have been well below atmospheric.

Residual moisture and mda values for the rubber-stoppered materials are given in table 9.16.

These data show that in both preparations, 80/120 and 82/253, there was an increase in residual moisture and in mda over the four weeks of storage at 37°C. The degradation of the material may have been due to an oxidation similar to that seen in the stored BCT preparations in Chapter 7.3, and may have been facilitated by ingress of atmospheric moisture and oxygen. In contrast, the data shown in table 9.15 indicate that the materials in the fusion-sealed vampoules was unchanged in residual moisture or mda content throughout. The mechanism of the degradation of these preparations is unlikely, therefore, to have been dependent upon an oxidative process.

TABLE 9.16 RESIDUAL MOISTURE AND MALONDIALDEHYDE IN 80/120 AND 82/253 IN RUBBER STOPPERED ENCLOSURES, AT 37°C.

	residual moisture %		mda (mmole/dm^{-3})	
	80/120	82/253	80/120	82/253
baseline	1.83	1.90	11	17.5
2 weeks	1.89	1.99	15	27
4 weeks	1.97	2.11	19	39.5

Discussion - Lyophilised thromboplastins stored in fusion-sealed vampoules showed significantly better stability than either rubber-stoppered vials or vampoules in an accelerated degradation study at 37°C. Since the thromboplastin was sequestered away from atmospheric water vapour and oxygen, it may be assumed that neither of these contributed to the degradation of the material. There will have been a small quantity of residual moisture from the outset, 1.83% for batch 80/120 and 1.9% for 82/253. These values, as seen in Chapter 9.1, were similar to seventeen other thromboplastins which were stable throughout long-term storage at -20°C.

Certain questions arise from these observations - firstly, what made the rubber-stoppered preparations degrade so quickly? The answer to this may be that the stoppered vials and, to a larger extent, the stoppered vampoules could not prevent the ingress of water vapour and oxygen which may have aided the degradative process. From the data on mda levels, this process seems to have been an oxidative one. Hopkins (1976) described the difficulties faced by manufacturers of rubber stoppers for freeze-dried preparations. A rubber stopper formula may comprise a polymer, a

reinforcing agent, an activator, a curing agent, pigments and processing aids such as plasticizers and softeners. The rubber stoppers used in the freeze-drying experiments reported here are made of butyl rubber, i.e. the polymeric ingredient is a copolymer of 97% isobutylene and 3% isoprene (natural rubber). Aluminium chloride is the catalyst employed in the polymerisation process. Plasticizers such as phthalate esters or ethylene glycol may be added to increase flexibility of the final product. To reduce friction in mixing these materials, softeners such as vegetable oils may be added. These do not enter into chemical combination with the polymer. Butyl rubber has a useful low temperature tolerance with a glass transition point around $-75^{o}C$. Other polymers, such as neoprene (polychloroprene) have a glass transition around $-40^{o}C$ while silicone (a large group of siloxane polymers) has a slightly lower glass transition of about $-50^{o}C$, but is more permeable to gases. Steinherz (1963) gives values for permeability of 100 x 10^{-8} cm^3 sec^{-1} $inch^{-1}$ of gasket, for silicone and 0.25 x 10^{-8} cm^3 sec^{-1} $inch^{-1}$ for butyl rubber. The selection of butyl rubber as the material of choice for freeze-drying stoppers was made with these considerations in mind.

It may be that the permeability of the stopper is increased at higher storage temperature. This is implied by the observations in Chapter 9.1 of long shelf life at $-20^{o}C$ of the same thromboplastins which last for only a few weeks at $37^{o}C$.

A further concern is that there may be an interaction of the thromboplastin and some volatile component of the rubber stopper

formulation. It is possible that such leaching from the stopper might occur at 37°C, but be so slow as to go unnoticed at -20°C. This is less likely to be the case, however, since higher residual mosture and mda levels in the degraded material lend weight to the idea of an oxidative process aided by permeating water vapours and oxygen.

It was noticed that the stopper/vampoule combination was less successful than the stopper/vial equivalent. It should be pointed out that vampoules are manufactured with their necks rather less rigourously finished than those of freeze-drying vials. Manufacturers apparently believe that since stoppering of vampoules will be a temporary measure only, it is not necessary to finish their necks to a very high standard. The consequence of this is that many vampoules will not seal in vacuo and it is possible that there will be admission of atmospheric vapour through ill fitting stoppers.

Greiff et al (1975) reported on the use of several synthetic elastomers such as neoprene, methacrylate and polyvinyl alcohol applied as an extra sealing layer to already sealed vials. Their best result was obtained with neoprene although the technique had many technical difficulties which rendered the process impractical for large batches of vials. Greiff et al (1975) and MacKenzie et al (1976) have commented on the adequacy of various methods of sealing of glass ampoules and Roth (1966) has described the advantages of the "draw-seal" method employed in the present experiments.

A second question, related to the thromboplastin in fusion-sealed vampoules, is what process is responsible for the degradation of the material? As already discussed, the components of this degradative process, or their precursors, are within the material from the outset. A sealed glass container allows no atmospheric gases to reach the dry thrombplastin. The low level of residual moisture favours the long term stability of freeze-dried thromboplastins at -20°C and degradation of the lipid moiety is ruled out by the low mda level in the degraded material. A mechanism involving proteins or interactions of proteins with sugars, e.g. the mechanism known as non-enzymatic browning may be involved.

This reaction, known as the Maillard reaction, is complex and incompletely understood. Its importance to the food industry and its physiological significance have been described and discussed, e.g. Labuza and Saltmarch (1981), Means and Min (1982). The first step in the process involves a condensation reaction between an amino group, contributed by a free amino acid or a protein, and the carbonyl group of a reducing sugar, resulting in the production of a Schiffs base and a molecule of water. The reaction is affected by factors such as the type and quantity of initial reactant species, pH, water content, and storage temperature. The total protein in freeze-dried thromboplastin reagents is in the range 3 - 4.5 g dm^{-3} whle glucose is present below 0.2 mmol dm^{-3} (Stevenson 1978). Means and Min (1982) have pointed out that only a very small fraction of most common sugars is normally present in aldehydic form e.g. 0.25% of fructose and <0.1% of glucose. These observations

reduce the likelihood of the Maillard reaction taking place on anything but a very limited scale. A further difficulty is that the pH of thromboplastin reagents is in the range 6 - 6.5 (Stevenson 1978) while the Maillard reaction is favoured by much higher pH values (due to increased reactivity of free amino groups). Saltmarch and Labuza (1982) reported that water can retard the rate of the initial glycosylamine reaction in which water is a product. These authors also point out the dilution of reactant species that would occur when water content is increased. This must be balanced, however, by the increased mobility of reactive species that would follow from decreased viscosity of the aqueous phase on dilution. Increased rates of reaction would result. Labuza and Saltmarch (1981) also reported increased rates of reaction when temperature was increased in a variety of test systems. It is possible therefore that the Maillard reaction has a role in the degradation of freeze-dried thromboplastin reagents. It is the case that aged thromboplastin reagents darken in colour and show increased absorbance at 420 nm (Stevenson 1980 - unpublished observation). This is consistent with the reported data on non-enzymatic browning. The values for residual moisture given in table 9.15 show no increase with time of storage but may simply indicate that the heat and weigh method may not be sensitive enough to detect any small increase in moisture due to the Maillard reaction.

Conclusions - Thromboplastin in rubber-stoppered vials and vampoules stored at 37^{o}C degrades in a few weeks, in a manner similar to that shown by liquid BCT stored at 4^{o}C (see Chapter 7.3). An oxidative process is responsible. These observations

suggest that permeability of rubber-stoppers may be an important feature in the degradation process. Thromboplastins stored at 37°C in fusion-sealed vampoules were stable for many months and finally degraded in a manner not consistent with oxidation of the lipid moiety. The WHO Secretariat (1965) declared that "In all cases, the sealing of ampoules should be by fusion of the glass. Sealing by rubber or plastic closures is unsatisfactory for long term preservation of international standards, reference preparations and reference reagents." The above experiments confirm this statement but only during high temperature storage. At -20°C storage there appears to be no disadvantage in using butyl rubber stoppers.

9.7 DISCUSSION

In the first experimental series it was shown that a series of batches of thromboplastin reagent, freeze-dried in a standardized manner, dried to a residual moisture value of 1.84% with a standard deviation of 0.18. This drying regime produced reagents with excellent long-term stability, which were calibrated in international multicentre exercises and adopted as International Reference Preparations of the EEC, WHO and ICSH.

Some modifications to the freeze-drying machinery were undertaken to examine the effect of liquid nitrogen cooling both in the sublimation stage and in the desorption stage of the process. A reduction in residual moisture was obtained when condenser temperature was lowered. As considerable quantities of liquid nitrogen were required for this cooling the method was not considered practical. A finding that was of great interest was that the surface area of the condenser could be as little as 19% of shelf area and show no evidence of decreased efficiency. In fact, the opposite was the case, since the reagent dried to significantly lower residual moisture when the liquid nitrogen trap was used. A more economical use of liquid nitrogen was possible, however, as shown in Chapter 9.3 when the P_2O_5 trap was replaced by a device cooled by liquid nitrogen. This device proved successful in providing an efficient trap for desorbing vapour. It was easier to clean and also avoided the use of P_2O_5, an unpleasant reagent to handle both in its dry state and when quenched.

Chapter 9.4 was designed to test the freeze-drying machine for differences from front to back of a shelf, from middle to edge of a shelf, from top to bottom in an array of shelves and from chamber to chamber in the machine. Testing of residual moisture from some 114 different sites within the system provided the data. While some statistically significant differences were noted, it was clear that the data were distributed in a very narrow range (mean value 1.81%, standard deviation 0.01) and within the calculated coefficient of variation for the method.

In the next section a description was given of the preparation of some thromboplastin reagents to serve as reference preparations. The dispensing of these materials, in one case requiring a specially designed dispensing device, is described together with fusion sealing of the WHO batch 82/253. The findings for CV of dispensed weight of reagent and for prothrombin time testing are given for each reagent. These results are encouraging and lend weight to the idea that intrinsic variation in the dispensing and freeze-drying machinery is very small. Long term studies of stability are presented which show that 79/099 and 84/441 remain stable to date. Residual moisture values for all three reagents were lower than 2% which may have been a contributing factor to their long term stability and the ISI values indicate that their sensitivity to the coumarin-induced defect is good. In the final section a comparison is made of the stability of thromboplastin reagents stored in rubber-stoppered vials and fusion-sealed glass. Data are presented that indicate that different degradative mechanisms must operate in the two types of enclosure stored at elevated temperature. The

rubber-stoppered vial is not a suitable enclosure for long-term storage of thromboplastin at high temperature. Stevenson (1978) found that the change in thromboplastin that occurred during storage in the freeze-dried state at 56°C was not the same as the changes that occured in liquid reagent stored at 4°C. The current experiment reveals that more than one mechanism of degradation also operates at 37°C. For practical purposes, however, it is clear that thromboplastin reagents stored in rubber-stoppered vials at -20°C have a long shelf life. High temperature storage must be avoided. Reagents stored in fusion-sealed glass can tolerate higher temperature storage but eventually degrade in a fashion that does not involve the oxidative processes reported for the reagent at lower temperatures.

CHAPTER 10 CONCLUSION

Original work presented includes a study, by electron microscopy of the tissue thromboplastin reagent (British Comparative Thromboplastin). Its changing morphology over a period of 102 months was revealed.

A concomitant alteration in lipid class composition and fatty acid distribution was shown and an increase in malondialdehyde with time of storage was noted also, observations strongly indicative of an oxidative process.

An electron microscopic study on some widely used partial thromboplastin reagents demonstrated for the first time that these are composed of liposomes. These were sized and their electrophoretic mobility determined. Their lipid composition was elucidated and a search for correlations between performance in the clotting test and their physical and chemical properties was carried out. A number of significant correlations were noted e.g. the APTT of normal plasma shortened with increasing relative concentration of PS.

In consequence, liposomes were manufactured from individual pure lipids to determine whether the brain and soya bean extracts that comprised the APTT reagents could be mimicked in their activity by simple mixtures of pure components. First a vehicle liposome was constructed. This showed neither pro- nor anticoagulant properties

in the APTT test system and was used as a vehicle for testing other phospholipids.

Of those phospholipids tested, PS was unique in its ability to shorten the APTT, i.e. to increase the procoagulant activity of the test liposome. Other phospholipids showed little or no capacity to confer procoagulant activity. PI was of interest since it possessed some anticoagulant properties. Sensitivity to heparin could also be adjusted by controlling the relative concentration of PS. Liposomes containing PS accelerated the APTT of plasmas containing a lupus-like anticoagulant while having no effect on APTT prolonged by other types of inhibitor, factor deficiencies, or by oral anticoagulants. Support is offered for the idea that the lupus anticoagulant acts by competing with coagulation factors for sites on procoagulant lipid surfaces.

Finally, some investigations were made in the freeze-drying of tissue thromboplastin reagents. The mean value for residual moisture of 19 batches of the lyophilised thromboplastin was found to be 1.84%. When the freeze-drying machine was modified by fitting a liquid nitrogen-cooled condenser, operating at very low temperatures, drying efficiency was improved with reduction of residual moisture coinciding with reduction of condenser temperature. Condenser surface area in these experiments was approximately 19% of shelf area, a much lower proportion than was previously thought practicable. The P_2O_5 trap was replaced in later experiments by a liquid nitrogen-cooled apparatus. This also reduced residual moisture and proved more economical of liquid

nitrogen. The freeze-drying machinery was found to be free of any intrinsic variation. Vial positioning from front-to-back or edge-to-middle on a shelf, or between shelves in one chamber, or between chambers, did not give rise to any variation outside the error of the residual moisture method.

Steps taken in the freeze-drying of three International Reference Preparations of thromboplastin of the World Health Organisation, the Bureau Communautaire de Reference of the EEC, and the International Committee for Standards in Haematology are described. Consideration was given to their long-term stability. A comparison of rubber-stoppered vials with fusion-sealed vampoules revealed a marked improvement in stability of tissue thromboplastin reagents sealed in glass. This improvement was seen at 37°C but not at -20°C, when rubber-stoppered vials appeared to be equally suitable enclosures.

It is hoped that these contributions to the knowledge of thromboplastins may be of some value in the still active quest for universally acceptable standards for thromboplastin reagents.

REFERENCES

Akkerman J W N, Nieuwenhuis H K, Mommersteeg-Leautaud M E, Gorter G, Sixma J J (1983) Br J Haematol 55 135

Akkerman J W N (1988) Introduction to blood platelets. In: Coagulation and Lipids ed: Zwaal R F A. CRC Press, Boca Raton p 49

Alderson M R, Poller L, Thomson J M (1970) J Clin Pathol 23 281

Altmann R (1890) Die Elementaro oranismen und ihre Berziehungen zuden Lellen, Veit & Co, Leipzig

Andersson L O, Brown J E (1981) Biochem J 200 161

Andersson L O, Phuc Thuy L, Brown J E (1981) Thromb Res 23 481

Astrup T, Buluk K (1963) Circ Res 13 253

Astrup T (1965) Thromb Diath Haemorrh 14 401

Atha D H, Stephens A W, Rimon A, Rosenberg R D (1984) Biochemistry 23 5801

Atkins E D T (1975) Heparin Symposium, Kings College Hospital, London, 20 April

Aurora T S, Li W, Cummins H Z, Haines T H (1985) Biochim Biophys Acta 820 250

Babson A L, Babson S R (1974) Am J Clin Path 62 856

Bach R, Nemerson Y, Konigsberg W (1981) J Biol Chem 256 824

Bach R, Gentry R, Nemerson Y (1986) Biochemistry 25 4007

Bach R (1988) CRC Crit Rev Biochem 23 339

Bailey N T J (1968) Statistical Methods in Biology. English Universities Press, London, p 100

Bangham A D, Heard D H, Flemans R, Seaman G V (1958) Nature 182 642

Bangham A D (1961) Nature 192 1107

Bangham A D (1961b) Nature 192 1197

Bangham A D, Horne R W (1964) J Mol Biol 8 660

Bangham D R (1966) Thromb Diath Haemorrh Supp 20: 341

Bangham D R, Biggs R, Brozovic M, Denson K W E (1973) Thromb Diath Haemorrh 29 228

Bangham A D, Hill M W, Miller N G (1974) Methods Membr Biol 1 1

Barbaree J M, Smith S J (1981) Cryobiology 18 528

Barkhan P, Silver M J, O'Keefe L M (1961) In: "Blood Platelets" eds: Johnson S A, Monto R W, Rebuck J W, Horn R C. Little Brown, Boston, Massachusetts, p 303

Barrowcliffe T W, Gray E (1981) Thromb Haemostas 46 634

Barber A A, Wilbur K M (1959) Radiat Res 10 167

Barsukov L I, Victorov A V, Vasilenko I A (1980) Biochim Biophys Acta 598 153

Basu D, Gallus A, Hirsh J (1972) New Eng J Med 287 324

Baumann A (1913) Biochem Z 54 30

Behringwerke Aktiengesellschaft (1984) European Patent no. 0123883

Bell R L, Majerus P W (1970) J Biol Chem 245 255

Benveniste J, Henson P M, Cochrane C G (1972) J Exp Med 136 1356

Benveniste J, Tence M, Varenne P, Bidault J, Boullet C, Polonsky J (1979) C R Acad Sci (Paris) 289 1037

Berden J A, Barker R W, Radda G K (1975) Biochim Biophys Acta 375 186

Bergelson L D (1979) Methods Membr Biol 9 275

Berndt M C, Phillips D R (1981) Platelet membrane proteins: composition and receptor function. In: Platelets in Biology and Pathology 2 ed: Gordon J L. Elsevier/North Holland, Amsterdam, p 44

Bernheim B V (1910) J A M A 55 283

Bertina R M, Alderkamp G H J, De Nooy E (1981) Thrombos Haemostas 46 88

Bertina R M, van den Besselaar, Bom V J J (1988) Tissue thromboplastin and the initiation of coagulation. In: Coagulation and Lipids, ed: Zwaal R F A. CRC Press, Boca Raton, p 131

Bevers E M, Comfurius P, van Rijn J L M L, Hemker H C, Zwaal R F A (1982) Eur J Biochem 122 429

Bevers E M, Comfurius P, Zwaal R F A (1983) Biochim Biophys Acta 736 57

Bevers E M, Zwaal R F A (1988) Role of platelets in factor X and prothrombin activation. In: Coagulation and Lipids, ed: Zwaal R F A. CRC Press, Boca Raton, P 16

Biggs R, Denson K W E (1966) Thromb Diath Haemorrh Supp 20: 345

Biggs R, Denson K W E (1967) British Medical J 1 84

Billah M M, Lapetina E G (1983) Proc Natl Acad Sci USA 80 965

Bingham J B, Meyer O O, Pohle F J (1941) J Amer Med Sci 202 653

Bitman J, Wood D L, Ruth J M (1981) J Liq Chromatog 4 1007

Bjorklid E, Storm E, Prydz H (1973) Biochem Biophys Res Commun 55 969

Bjorklid E, Storm-Mathisen J, Storm E, Prydz H (1977) Thromb Haemostas 37 91

Bjorklid E, Storm E (1977) Biochem J 165 89

Bjorklid E, Holm T, Østerud B (1987) Thromb Res 45 609

Bligh E G, Dyer W J (1959) Can J Biochem Physiol 37 911

Blok M C, van der Neut-Kok E C M, van Deenen L L M, de Gier J (1975) Biochim Biophys Acta 406 187

Bloom E J, Abrams D I, Rodgers G (1986) J A M A 256 491

Bloom J W, Nesheim M E, Mann K G (1979) Biochemistry 18 4419

Bom V J J, Bertina R M (1985) Thromb Haemostas 54 215

Boggs J M, Moscarello M A (1978) Biochim Biophys Acta 515 1

Bona R, Lee E, Rickles F (1987) Thromb Res 48 487

Bowie E J, Thompson J H, Pascuzzi C A, Owen C A (1963) J Lab Clin Med 62 416

Box G E P, Cox D R (1964) J Royal Statistical Soc Series B 26 211

Brandt J T, Triplett D A (1981) Am J Clin Pathol 76 (suppl) 530

Brandt J T, Triplett D A, Musgrave K, Orr C (1987) Arch Pathol Lab Med 111 120

Bretscher M S, Raff M C (1975) Nature 258 43

Brinkhous K M, Langdell R D, Penick G D, Graham J B, Wagner R H (1954) J Amer Med Assoc 154 481

Broze G J (1982) J Clin Invest 70 526

Broze G J, Leykam J E, Schwartz B D, Miletich J P (1985) J Biol Chem 260 10917

Buege J A, Aust S D (1978) Meth Enzymol 52 302

Butt H R, Allen E V, Bollman J L (1941) Proc Mayo Clinic 16 388

Bull R K, Jevon S, Barton P G (1972) J Biol Chem 247 2747

Campbell H A, Link K P (1941) J Biol Chem 138 21

Campbell L L (1954) J Amer Pharm Assoc 43 359

Carman P C (1948) Trans Farad Soc 44 529

Carreras L O, Machin S J, Deman R, Defreyn G, Vermylen J, Spitz B, van Assche A (1981) Lancet 1 244

Carson S D, Bach R, Carson S M (1985) Blood 66 152

Carson S D, Henry W M, Haley L, Byers M, Shows T (1985) Cytogenet Cell Genet 40 600

Casu B (1985) Adv Carb Chem Biochem 43 51

Chap H, Douste-Blazy L (1974) Eur J Biochem 48 351

Chap H J, Zwaal R F A, van Deenen L L M (1977) Biochim Biophys Acta 467 146

Chap H, Mauco G, Simon M F, Benveniste J, Douste-Blazy L (1981) Nature 289 312

Chapman D, Williams R M, Ladbrooke B D (1967) Chem Phys Lipids 1 445

Chapman D (1984) Physicochemical properties of phospholipids and lipid-water systems. In: Liposome Technology Vol 1 Preparations of Liposomes, ed: Gregoriadis G. CRC Press, Boca Raton, p 1

Chargaff E, Bendich A, Cohen S S (1944) J Biol Chem 156 161

Chargaff E (1948) J Biol Chem 173 253

Chio K S, Tappel A L (1969) Biochemistry 8 2821

Chiu D, Lubin B, Roelofsen B, van Deenen L L M (1981) Blood 58 398

Chong C S, Colbow K (1976) Biochim Biophys Acta 436 260

Ciborowsky J, Surgiewiez J (1961) Przem Chem 40 646

Clarke N, O'Meara R A Q (1966) Br J Haematol 12 536

Clouse L H, Comp P C (1986) N Engl J Med 314 1298

Cohen A J, Philips T M, Kessler C M (1986) Ann Intern Med 104 175

Cohen G, Hochstein P (1961) Science 134 1574

Comfurius P, Bevers E M, Zwaal R F A (1983) Biochem Biophys Res Commun 117 803

Comfurius P, Bevers E M, Zwaal R F A (1985) Biochim Biophys Acta 815 43

Conley C L, Hartmann R C (1952) J Clin Invest 31 621

Constantinides P (1967) Arch Pathol 83 422

Coots M C, Miller M A, Glueck H I (1981) Thromb Haemostas 46 734

Cooper M R, Cohen J H, Huntley C C (1974) Blood 43 493

Cornell B A, Fletcher G C, Middlehurst T J, Separovic F (1982) Biochim Biophys Acta 690 15

Cornell B A, Middlehurst J, Separovic F (1986) Faraday Discuss Chem Soc 81 163

Cullis P R, de Kruijff B (1979) Biochim Biophys Acta 559 399

Cullis P R, Verkleij A J (1979) Biochim Biophys Acta 552 546

Cullis P R, Hope M J, de Kruijff B, Verkleij A J, Tilcock C P S (1985) Structural properties and functional roles of phospholipids in biological membranes. In: Phospholipids and Cellular Regulations Vol 1, ed: Kuo J F. CRC Press, Boca Raton, p 1

Daemen F J, van Arkel C, Hart H Ch, van der Drift C, van Deenen L L M (1965) Thromb Diath Haemorrh 13 194

Dahlback B, Stenflo J (1980) Eur J Biochem 107 331

Dahlback B, Nilsson I M, Frohm B (1983) Blood 62 218

Dahlback B (1986) J Biol Chem 261 12022

Dahle L K, Hill E G, Holman R T (1962) Arch Biochem Biophys 98 253

Dalaker K, Kaplun A, Lyberg T, Prydz H (1983) Gynaecol Obstet Invest 15 351

Dalaker K, Prydz H (1983) Thromb Haemostas 50 831

Dam H, Doisy E A (1964) Nobel Lectures: physiology or medicine, 1942-1962, Elsevier, N York

Davie E W, Ratnoff O D (1964) Science 145 1310

Davie E W, Fujikawa K, Kisiel W (1979) Adv Enzymol 48 277

Davies G E, Palek J (1982) Blood 59 502

De Koning G A, Silk J (1963) J Am Oil Chem Soc 40 165

De Kruijff B, Verkleij A J, van Echfeld C J A, Gerritsen W J, Mombers C, Noordam P C, De Gier J (1979) Biochim Biophys Acta 555 200

Demarco L, Girolami A, Zimmerman T S, Ruggeri Z M (1985) Proc Natl Acad Sci USA 82 7424

Demel R A, De Kruijff B (1976) Biochim Biophys Acta 457 109

Demopoulos C A, Pinckard R N, Hanahan D J (1979) J Biol Chem 254 9355

Denson K W E (1966) Thromb Diath Haemorrh Supp 20: 363

Denson K W E, Bonnar J (1973) Thromb Diath Haemorrh 30 471

De Roissart H, Laederich T (1966) In Lyophilisation ed: Rey L. Hermann, Paris, p 55

Deutsch E, Irsigler K, Lomoschitz H (1964) Thromb Diath Haemorrh 12 12

Discipio R G, Davie E W (1979) Biochemistry 18 899

Drake T A, Morissey J H, Edgington T S (1989) Am J Pathol 134 1087

Drakenberg T, Fernlund P, Roepstorff P, Stenflo H (1983) Proc Natl Acad Sci USA 80 1802

Dressler V, Haest C W M, Plasa G, Deuticke B, Erusalimsky J D (1984) Biochim Biophys Acta 775 189

Elias M, Elder A (1984) Arch Intern Med 144 510

Elser W J (1934) US Patent no. 1970956

Elser W J, Thomas R A, Sleffern G I (1935) J Immunol 28 433

Esmon C T, Jackson C M (1974) J Biol Chem 249 7782

Esmon C T, Owen W G, Jackson C M (1974) J Biol Chem 249 7798

Esmon C T (1979) J Biol Chem 254 964

Esmon C T, Esmon N L, Harris K N (1982) J Biol Chem 257 7944

Exner T, Rickard K A, Kronenberg H (1978) Br J Haematol 40 143

Exner T, Rickard K A, Kronenberg H (1980) Pathology 12 559

Falk E (1983) Br Heart J 50 127

Fasco M J, Principe L M (1982) J Biol Chem 257 4894

Feinstein D I, Rapaport S I (1972) Prog Haemostasis Thromb 1 75

Feinstein M B, Rodan G A, Cutler L S (1981) Cyclic AMP and calcium in platelet function. In: Platelets in Biology and Pathology, ed: Gordon J L. Elsevier/North Holland, Amsterdam, p 437

Fiedel B A, Osmand A P, Gewurz H (1976) Nature 263 687

Fildes F J T (1981) Liposomes: the industrial viewpoint In: Liposomes: From physical structure to therapeutic applications, ed: Knight C G. Elsevier, Amsterdam, p 465

Finkelstein M C, Weissmann G (1979) Biochim Biophys Acta 587 202

Flosdorf E W, Mudd S (1935) J Immunol 29 389

Flosdorf E W, Stokes F J, Mudd S (1940) J A M A 115 1095

Forman S D, Nemerson Y (1986) Proc Natl Acad Sci USA 83 4675

Forrester J S, Litvack F, Grundfest W, Hickey A (1987) Circulation 75 505

Fowler J, Cohen L (1986) Statistics for Ornithologists. British Trust for Ornithology Guide 22, p 140

Fox J E B (1985) J Biol Chem 260 11970

Fox J E B (1985) J Clin Invest 76 1673

Fox J E B, Goll D E, Reynolds C C, Phillips D R (1985) J Biol Chem 260 1060

Franck P F H, Bevers E M, Lubin B H, Comfurius P, Chiu D T Y, Op den Kamp J A F, Zwaal R F A, van Deenen L L M, Roelofsen B (1985) J Clin Invest 75 183

Franks F (1986) Cryoletters 7 207

Friedman M, van den Bovenkamp G J (1966) Am J Pathol 48 19

Fromherz P (1983) Chem Phys Lett 94 259

Fujikawa K, Heimark R L, Kurachi K, Davie E W (1980) Biochemistry 19 1322

Furie B, Furie B C (1988) Cell 53 505

Galdal K S, Lyberg T, Evensen S A, Nilsen E, Prydz H (1984) Biochem Pharmac 33 2723

Galliard T (1973) Phospholipid metabolism in photosynthetic plants. In: Form and Function of Phospholipids, eds: Ansell G B, Hawthorne J W, Dawson R M C. Elsevier, Amsterdam, p 253

Geczy C L, Hopper K E (1981) J Immunol 126 1059

Gerritsen W J, Van Zoelen E J, Verkleij A J, De Kruijff B, van Deenen L L M (1979) Biochim Biophys Acta 551 248

Girolami A, Vicarioto M, Ruzza G, Cappellato G, Vergolani A (1985) Acta Haematol 73 31

Gonmori H, Takeda Y (1976) Thromb Haemostas 36 90

Gorter E, Grendel F (1925) J Exp Med 41 439

Gounaris K, Sen A, Brain A P R, Quinn P J, Williams W P (1983) Biochim Biophys Acta 728 129

Gratzner W B (1981) Biochem J 198 1

Greaves R I N, Adair M E (1936) J Hyg (Camb) 36 507

Greaves R I N, Adair M E (1939) J Hyg (Camb) 39 413

Greaves R I N (1946) Nature, Lond 153 483

Greenberg C S, Shuman M A (1984) J Biol Chem 259 14721

Greengard J S, Griffin J H (1985) Biochemistry 23 6863

Gregoriadis G, Davis C (1979) Biochem Biophys Res Commun 89 1287

Gregory D W, Pirie B J S (1973) J Microsc Oxford 99 251

Greiff D, Melton H, Rowe T W G (1975) Cryobiology 121

Griffith M J (1982) J Biol Chem 257 7360

Griffith M J (1983) Proc Natl Acad Sci USA 80 5460

Grob K, Grob G (1979) High Resolution Chromatog and Chromatog Commun 2 109

Gruner S M, Leuk R P, Janoff A S, Ostro M J (1985) Biochemistry 24 2833

Guha A, Bach R, Konigsberg W H, Nemerson Y (1986) Proc Natl Acad Sci USA 83 299

Guiot P, Bandjuin P, Gotfredsen C (1980) J Microsc 120 159

Haest C W M, Plasa G, Kamp D, Deuticke b (1978) Biochim Biophys Acta 509 21

Hallam T J, Sandez A, Rink T J (1984) Biochem J 218 819

Hannan R S and Lea C H (1952) Biochem J 52 105

Harris K W, Esmon C T (1985) J Biol Chem 260 2007

Hasegawa H, Nagata H, Murao M (1977) Thromb Haemostas 37 541

Hathaway W E, Assmus S L, Montgomery R R, Dubansky A S (1979) Am J Clin Pathol 71 22

Helfrich W (1974) Phys Lett 50A 115

Hemker H C, Esnouf M P, Hemker P W, Swart A C, MacFarlane R G (1967) Nature 215 248

Hemker H C, Kahn M J P (1967) Nature 215 1201

Hemker H C, Hemker P W (1969) Proc R Soc Ser B 173 411

Hemker H C (1987) The mode of action of heparin in plasma. In: Thrombosis and Haemostasis, eds: Verstraete M et al. ISTH and Leuven University Press, Leuven, p 17

Hemker H C (1988) Introduction to the mechanism of blood coagulation. In: Coagulation and Lipids, ed: Zwaal R F A. CRC Press, Boca Raton, p 1

Hendrix H, Lindhout T, Mertens K, Engels W, Hemker H C (1983) J Biol Chem 258 3637

Hermans J (1984) The European Community Bureau of Reference Calibration Study. In: Thromboplastin Calibration and Oral Anticoagulant Control, eds: van den Besselaar A M H P, Gralnick H R, Lewis S M. Martinus Nijhoff Publishers, Boston, p 41

Hetland Ø, Brovold A B, Holme R, Gaudernack G, Prydz H (1985) Biochem J 228 735

Hibbard L S, Mann K G (1980) J Biol Chem 255 638

Hiemark R L, Schwartz S (1983) Biochem Biophys Res Commun 111 723

Higgins D L, Mann K G (1983) J Biol Chem 258 6503

Hills M, Ingram G I C (1973) Br J Haematol 25 445

Hjort P F (1957) Scand J Clin Lab Invest 9 suppl 27 1

Hochstein P (1981) Israel J Chem 21 52

Hoffman J J M L, Meulendijk P N (1978) Thromb Haemostas 39 640

Hook M, Kjellen L, Johansson S, Robinson J (1984) Ann Rev Biochem 53 847

Hope M J, Janoff A S (1986) J Biol Chem 261 9672

Hopkins G H (1976) Develop Biol Standard 36 139

Hougie C (1959) Proc Soc Exp Biol (NY) 101 132

Howell W H (1912) Am J Physiol 31 1

Huang C H (1969) Biochemistry 8 344-352

Huang C H, Charlton J P (1971) J Biol Chem 246 2555

Hvatum M, Prydz H (1966) Biochim Biophys Acta 130 92

Ingram G I C (1979) Thromb Haemostas 42 1135

Inoue K (1974) Biochim Biophys Acta 339 390

Israelachvili J N, Mitchell D J (1975) Biochim Biophys Acta 389 13

Israelachvili J N, Mitchell D J, Ninham B W (1976) J Chem Soc, Faraday Trans 72 1525

Israelachvili J N, Mitchell D J, Ninham B W (1977) Biochim Biophys Acta 470 185

Israelachvili J N, Marcelja S, Horn R J (1980) Q Rev Biophys 13 121

Jaffe E A (1987) Hum Pathol 18 234

Janson T L, Stormorken H, Prydz H (1984) Haemostasis 14 440

Jennings W (1980) "Gas Chromatography with Glass Capillary Columns" Academic Press, N York, p 134

Jesty J, Morrison S A (1983) Thromb Res 32 171

Jobin F, Esnouf M P (1967) Biochem J 102 666

Johnsen U L H, Lyberg T, Galdal K J, Prydz H (1983) Thromb Haemostas 49 69

Jordan R E, Oosta G M, Gardner W T, Rosenberg R D (1980) J Biol Chem 255 10081

Josso F, Prou-Wartelle O (1965) Thrombos Diath Haemorrh. suppl 17 35

Kadish J L, Wenc K M, Dvorak H F (1983) J N C I 70 551

Kane W H, Majerus P W (1982) J Biol Chem 257 3963

Karpatkin S (1977) Composition of platelets. In: Haematology, eds: Williams W J, Beutler E, Erslev A J, Rundles R W. McGraw Hill, New York, p 1176

Kelsey. P.R., Stevenson. K.J., Poller. L. (1984) Thromb Haemostas 52 172

Kendal M G, Stuart A (1961) The Advanced Theory of Statistics 2. Charles Griffin, London

Kirk J E (1962) Proc Soc Exp Biol Med 109 890

Kirkland J J, Yan W W, Szoka F C (1982) Science 215 296

Kirkwood T B L (1983) Thromb Haemostas 49 238

Kirkwood T B L (1984) General aspects of thromboplastin calibration. In: Thromboplastin Calibration and Anticoagulant control, eds: van den Besselaar A M H P, Gralnick H R, Lewis S M. Martinus Nijhoff Publishers, Boston, p 11

Kisiel W, Canfield W M, Ericsson L H (1977) Biochemistry 16 5824

Kisiel W (1979) J Clin Invest 64 761

Kiss J (1974) Thromb Diath Haemorrh 33 20

Koepke J A (1975) Am J Clin Path 63 990

Koepke J A (1977) Quality Control in Laboratory Medicine, eds: Henry J B, Geiger J C. Masson, New York, p 157

Kramers H, Stemerding S (1951) Appl Sci Res A3 73

Kuhn R, Klesse P (1957) Naturwissenschaften 44 352

Labuza T P, Saltmarch M (1981) The non-enzymatic browning reaction as affected by water in foods. In: Water Relations in Foods 11, ed: Rockland L. Academic Press, New York, p 605

Lafer E M, Rauch J, Andrzejewski Ch, Mudd D, Furie B, Furie Br, Schwartz R S, Stollar B (1981) J Exp Med 153 897

Lajmanovich A, Hudrey-Clergeon G, Freyssinet J M, Margueri G (1981) Biochim Biophys Acta 678 132

Lam-Po Tang P R L C, Poller L (1975) Thromb Diath Haemorrh 34 419

Langdell R D, Wagner R H, Brinkhous K M (1953) J Lab Clin Med 41 637

Lapetina E G, Cuatrecasas P (1979) Biochim Biophys Acta 573 394

Lasic D D (1982) Biochim Biophys Acta 692 5;01

Lawler J (1986) Blood 67 1197

Leck I, Gowland E, Poller L (1975) Thromb Diath Haemorrh 34 419

Lee A G (1975) Prog Biophys Molec Biol 29 3

Leung L L K (1984) J Clin Invest 74 1764

Levy G A, Schwartz B S, Curtiss L K, Edgington T S (1981) J Clin Invest 67 1614

Lindahl U, Backstrom G, Hook M, Thunberg L, Fransson L A, Linker A (1979) Proc Natl Acad Sci USA 76 3198

Liu D T H, McCoy L E (1975) Thromb Res 7 199

Loeliger E A, Lewis S M (1982) Lancet ii 318

Lubin B, Chiu D, Bastacky J, Roelofsen B, van Deenen L L M (1981) J Clin Invest 67 1643

Luna E J, McConnell H M (1977) Biochim Biophys Acta 470 303

Lyberg T, Hetland O, Prydz H (1982) Thromb Haemostas 47 154

Lyberg T, Galdal K S, Evensen S A, Prydz H (1983) Br J Haematol 53 85

MacFarlane R G (1964) Nature 202 498

MacKenzie A P, Luyet B J (1964) Cryobiology (suppl) 1 17

MacKenzie A P, Welkie D G, Lagally M G, Pace M, Elliott F I (1976) Develop Biol Standard 36 151

Mannucci P M, Cauciani M T, Mari D, Meucci P (1979) Scand J Haematol 22 423

Mannucci P M (1982) A multicentre evaluation of partial thromboplastin time reagents in the detection of mild haemophiliacs. In: Standardisation of Coagulation Assays: an overview, ed: Triplett D A. CAP, Skokie, Illinois, p 165

Magnusson S, Sottrup-Jenssen L, Petersen T E (1975) FEBS Lett 44 189

Marcus A J, Spaet T H (1958) J Clin Invest 37 1836

Marcus A J, Ullman H L, Safier L B, Ballard H S (1962) J Clin Invest 41 2198

Marcus A J (1965) Thromb Diath Haemorrh Suppl 17 85

Marcus A J, Zucker-Franklin D, Safier L B, Ullman H L (1966) J Clin Invest 45 14

Marcus A J (1978) J Lipid Res 19 793

Margolis J (1957) J Physiol 137 95

Margolis J (1961) Aust J Exp Biol 39 249

Marinetti (1964) in New Biochemical Separations, Van Nostrand, Princeton, New Jersey, p 339

Marlar R A, Griffin J H (1981) Ann NY Acad Sci 370 325

Mason J T, Huang C H (1978) Ann NY Acad Sci 308 29

May J C, Grim E, Wheeler R M, West J (1982) J Biol Standard 10 249

May J C, Wheeler R M, Grim E (1989) Cryobiology 26 277

Mayer L D, Nelsestuen G L, Brockman H L (1983) Biochemistry 22 316

Maynard J R, Dreyer B E, Stemerman M B, Pitlick F A (1977) Blood 50 387

Mead J F (1982) Lipid Peroxides in Biology and Medicine, Academic Press, N York

Mead J F (1986) In: Lipids: Chemistry, Biochemistry and Nutrition. Plenum Press, N York, p 369

Means G E, Min K C (1982) Diabetes 31 (suppl 3) 1

Mellor J D, Munns A S (1968) Aust Refrig Air Cond Heat 22 (8) 20

Mellor J D (1978) Fundamentals of Freeze-drying, Academic Press, London

Mertens K, Giles A R, Briet E (1985) Thromb Haemostas 54 258

Mertens K, van Wijngaarden A, Bertina R M (1985) Thromb Haemostas 54 654

Metcalfe L D, Schmitz A A (1961) Anal Chem 33 363

McGowan E B, Yeo K T, Detwiler T C (1983) Arch Biochem Biophys 227 287

Middelkoop E, Lubin B H, Op Den Kamp J A F, Roelofsen B (1986) Biochim Biophys Acta 855 421

Miles L A, Ginsberg M H, White J C, Plow E F (1986) J Clin Invest 77 2001

Mills C A (1921) J Biol Chem 46 135

Mitchell D J, Ninham B W (1981) J Chem Soc, Faraday Trans 77 601

McMullen B A, Fujikawa K, Kisiel W (1983) Biochem Biophys Res Commun 115 8

Mombers C, de Gier J, Demel R A, Van Deenen L L M (1980) Biochim Biophys Acta 603 52

Morawitz P (1904) Ergebn Physiol 4 307

Morita T, Jackson C M (1986) J Biol Chem 261 4015

Morrissey J H, Fakhrai H, Edgington T S (1987) Cell 50 129

Nair V, Vietti D E, Cooper C (1981) J Am Chem Soc 103 3030

Nair V, Turner G A (1984) Lipids 19 804

Nair V, Cooper C, Vietti D E, Turner G A (1986) Lipids 21 6

Nawroth P, Kisiel W, Stern D (1985) Clin Haematol 14 531

Nawroth P P, Handley D, Stern D M (1986) Clin Haematol 15 293

Nelsestuen G L, Zytokovicz T H, Howard J B (1974) J Biol Chem 249 6347

Nelsestuen G L (1978) Fed Proc Fed Am Soc Exp Biol 37 2621

Nelsestuen G L, Broderius M (1977) Biochemistry 16 4172

Nelsestuen G L, Kisiel W, Discipio R G (1978) Biochemistry 17 2134

Nemerson Y (1968) J Clin Invest 47 72

Nemerson Y (1969) J Clin Invest 48 322

Nemerson Y, Pitlick F (1970) Biochemistry 9 5100

Nemerson Y, Gentry R (1986) Biochemistry 25 4020

Nesheim M E, Mann K G (1979) J Biol Chem 254 1326

Nesheim M E, Taswell J B, Mann K G (1982) Haemostasis 12 268

Nesheim M E, Tracy R P, Mann K G (1984) J Biol Chem 259 1447

Nicolson G L (1976) Biochim Biophys Acta 457 57

Nolf P (1908) Arch Int Physiol 6 1

O'Brien P F, North W R S, Ingram G I C (1981) Thromb Haemostas 45 162

O'Brien P J (1987) In: Autoxidation of Unsaturated Lipids, ed: Chan H W S. Academic Press, p 233

Oliver J M (1978) Am J Path 93 221

Ollivon M, Walter A, Blumental R (1986) Anal Biochem 152 262

Olson F, Hunt C A, Szoka F C, Vail W J, Papahadjopoulos D (1979) Biochim Biophys Acta 557 9

Op den Kamp J A F, Kauerz M T, van Deenen L L M (1975) Biochim Biophys Acta 406 169

Osterud B, Rapaport S I (1977) Proc Natl Acad Sci USA 74 5260

Otnaess H B, Holm T (1976) J Clin Invest 57 1419

Owren P A, Aas K (1951) Scand J Clin Lab Invest 3 201

Owren P A (1959) Lancet ii 754

Owen W G, Esmon C T, Jackson C M (1974) J Biol Chem 249 594

Papahadjopoulos D, Hougie C, Hanahan D J (1962) Proc Soc Exp Biol Med 111 412

Papahadjopoulos D, Hanahan D J (1964) Biochim Biophys Acta 90 436

Papahadjopoulos D, Watkins J C (1967) Biochim Biophys Acta 135 639

Papahadjopoulos D, Jacobson K, Nir S, Isac T (1973a) Biochim Biophys Acta 311 330

Papahadjopoulos D, Dowden M, Kimelberg H (1973b) Biochim Biophys Acta 330 8

Pearson E S, Hartley H O (1976) Biometrika Tables for Statisticians 1 Biometrika Trust, London

Peleg I, Muhlrad A, Eldor A, Groschel-Stewart U, Kahane I (1984) Arch Biochem Biophys 234 443

Pemberton J R (1976) Develop Biol Standard 36 191

Perret B, Chap H J, Douste-Blazy L (1979) Biochim Biophys Acta 556 434

Phillips M C, Chapman D (1968) Biochim Biophys Acta 163 301

Pitlick F A, Nemerson Y, Gottlieb A J, Gordon R G, Williams W J (1971) Biochemistry 10 2650

Pletcher C H, Cunningham M, Nelsestuen G L (1985) Biochim Biophys Acta 838 106

Ploplis V A, Castellino F J (1980) Effect of the biophysical properties of the phospholipid vesicle on the activation of bovine prothrombin. In: the Regulation of Coagulation, eds: Mann K G, Taylor F B. Elsevier, N York, p 121

Plow E F, Ginsberg M H (981) J Biol Chem 256 9477

Poller L (1964) British Medical J 2 565

Poller L (1967) Lancet i 491

Poller L, Thomson J M (1969) Br J Haematol 16 31

Poller L (1970) Assoc Clin Pathol Broadsheet no. 71

Poller L, Thomson J M, Palmer M K (1976) Lancet 2 842

Poller L, Thomson J M, Yee K F (1980) Br J Haematol 44 161

Poller L, Taberner D A, Sandilands D G, Galasko C S B (1982) Thromb Haemostas 47 50

Poller (1989) J Clin Pathol 42 1

Prydz H, Lyberg T, Deteix P, Allison A C (1979) Thromb Res 15 465

Prydz H, Pettersen K S (1988) Haemostasis 18 215

Pusey M L, Mayer L D, Jason Wei G, Bloomfield A, Nelsestuen G L (1982) Biochemistry 21 5262

Pusey M L, Nelsestuen G L (1984) Biochemistry 23 6202

Quick A J (1935) Am J Physiol 123 712

Radoff S, Danishefsky I (1984) J Biol Chem 259 166

Ratnoff O, Crum J D (1964) J Lab Clin Med 63 359

Rauch J, Tannenbaum M, Tannenbaum H, Ramelson H, Cullis P R, Tilcock C P S, Hope M J, Janoff A S (1986) J Biol Chem 261 9672

Renall M H (1913) Biochem 2 55 296

Resnick R M, Nelsestuen G L (1980) Biochemistry 19 3028

Rey L R (1964) Fundamental aspects of lyophilisation. In: Aspects Theoretiques et Industriels de la lyophilisation, ed: Rey L. Hermann, Paris, p 23

Rey L (1978) In: Fundamentals of Freeze-drying, ed: Mellor J D. Academic Press, London, foreword

Reynolds J A, Nozaki Y, Tanford C (1983) Anal Biochem 130 471

Rice-Evans C, Hochstein P (1981) Biochem Biophys Res Comm 100 1537

Rittenhouse S E (1983) Proc Natl Acad Sci USA 80 5417

Rivers R P A, Hathaway W E, Weston W L (1975) Br J Haematol 30 311

Roderick L M (1929) J Amer Vet Med Assoc 74 314

Rosenberg R D, Armand G, Lam L (1978) Proc Natl Acad Sci USA 75 3065

Rosenberg R D, Bauer K A, Marcum J A (1984) In: Reviews of Haematology, eds: Ambrus J L, Murano E. PJD Publications, Westbury, N York, p 131

Rosenberg R D, Rosenberg J S (1984) J Clin Invest 74 1

Rosing J, Tans G, Govers-Riemslag J W P, Zwaal R F A, Hemker H C (1980) J Biol Chem 255 274

Rosing J, van Rijn J L M L, Bevers E M, van Dieijen G, Comfurius P, Zwaal R F A (1985) Blood 65 319

Roth A (1966) Vacuum Sealing Techniques. Pergamon Press, London, p 73

Rouser G, Schloredt D (1958) Biochim Biophys Acta 28 81

Rouser G, White S G, Schloredt D (1958) Biochim Biophys Acta 28 71

Rouser G, O'Brien J, Heller D (1961) J Amer Oil Chemists Soc 38 14

Rouser G, Siakotos A N, Fleischer S (1966) Lipids 1 85

Rowe T W G (1964b) Fd Trade Rev 35 42

Rowe T W G, Snowman J W (1976) Edwards Freeze-drying Handbook, Edwards High Vacuum, Crawley, p 47

Sanders N L, Bajaj S P, Zivelin A, Rapaport S I (1985) Blood 66 204

Saltmarch M, Labuza T P (1982) Diabetes 31 (Suppl 3) 29

Satir B (1975) Scient Am 233 28

Saunders L, Perrin J, Gammack D B (1962) J Pharm Pharmacol 14 567

Scarpati E M, Wen D, Broze G J, Miletich J P, Flandermeyer R R, Siegel N R, Sadler J E (1987) Biochemistry 26 5324

Scheffe H (1959) The Analysis of Variance. Wiley and Sons, New York, p 55

Scherphof G, Morselt H, Regts J, Wilschut J C (1979) Biochim Biophys Acta 556 196

Schick P K, Kurica K B, Chacko G K (1976) J Clin Invest 57 1221

Schick P K (1978) J Lab Clin Med 91 802

Schick P K (1979) Seminars in Haematology 16 221

Schleider M A, Nachman R L, Jaffe E A, Coleman M (1976) Blood 48 499

Scofield F W (1922) Canad Vet Rec 3 74

Scott P R (1988) Vet Rec 123 373

Seigneuret M, Devaux P F (1984) Proc Natl Acad Sci USA 81 3751

Seligmann E B, Farber J F (1971) Cryobiology 8 138

Selser J C, Yeh Y, Baskin R J (1976) Biophys J 16 337

Shapiro G A, Huntziger S W, Wilson J E (1977) Am J Clin Path 67 477

Shapiro S S, Thiagarajan P (1982) Prog Haemostasis Thromb 6 263

Shumsky K (1958) Fd Technol 10 57

Sibley C A, Singer J W, Wood H J (1973) Am J Clin Path 59 581

Siegel S (1956) Nonparametric Statistics for the Behavioural Sciences. McGraw Hill Inc, Tokyo, p 202

Siess W, Siegel F L, Lapetina E G (1983) J Biol Chem 258 11236

Simon M F, Chap H, Douste-Blazy L (1984) FEBS Lett 170 43

Singer S J, Nicolson G L (1972) Science 175 720

Sinnhuber R O, Yu T C, Yu Te Chang (1958) Food Res 23 626

Skogen W F, Esmon C T, Cox A C (1984) J Biol Chem 259 2306

Solum N O, Olsen T M (1985) Biochim Biophys Acta 817 249

Spearman C (1904) Amer J Psych 15 88

Speer R J, Ridgway H (1962) Proc 9th Congr Intern Soc Haemtol, Mexico City

Spicer E K, Horton R, Bloem L, Bach R, Williams K R, Guha A, Kraus J, Lin T C, Nemerson Y, Konigsberg W H (1987) Proc Natl Acad Sci USA 84 5148

Stahl K, Therman H, Dame W R (1978) Haemostasis 7 242

Steinherz H A (1963) Handbook of High Vacuum Engineering. Reinhold Publishing Corporation, New York, p 759

Stenflo J, Fernlund P, Egan W, Roepstorff P (1974) Proc Natl Acad Sci USA 71 2730

Stern D M, Drillings M, Nossel H L, Hurlet-Jensen A, Lagamma K S, Owen J (1983) Proc Natl Acad Sci USA 80 4119

Stern D M, Bank I, Nawroth P P (1985) J Exp Med 162 1223

Stern D M, Nawroth P P, Kisiel W, Vehar G, Esmon C T (1985) J Biol Chem 260 6717

Stevenson K J (1978) Studies on the development of standardised reference thromboplastins for the laboratory control of anticoagulant therapy. M Phil Thesis. CNAA

Stevenson K J, Slater P J, Poller L (1980) Br J Haematol 44 495

Stevenson K J, Poller L (1982) Thromb Res 26 341

Stevenson K J, Easton A C, Thomson J M, Poller L (1983) Thromb Haemostas 50 601

Stevenson K J, Easton A C, Curry A, Thomson J M, Poller L (1986) Thromb Haemostas 55 250

Stevenson K J, Wilcox F L, Poller L, Burslem R W (1987) J Obstet Gyanecol 7 173

Stewart J C M (1980) Anal Biochem 104 10

Strumia M M, McGraw J J (1943) J Lab Clin Med 28 1140

Studer A (1946) Festschrift Emil Borell. Hoffman La Roche, Basel

Surgenor D M, Wallach D F H (1961) In Blood Platelets, eds: Johnson S A, Monto R W, Rebuck J W, Horn R C. Little Brown, Boston, Massachusetts, p 289

Suttie J W, Jackson C M (1977) Physiol Rev 57 1

Suzuki K, Dahlback B, Stenflo J (1982) J Biol Chem 257 6556

Suzuki K, Nishioka J, Hashimoto S (1983) J Biochem 94 699

Suzuki K, Nishioka J, Matsuda M, Murayama H, Hashimoto S (1984) J Biochem 96 455

Swinscow T D V (1976) Statistics at Square One. British Medical Association, London, p 33

Szoka F, Papahadjopoulos D (1980) Ann Rev Biophys Bioeng 9 467

Szoka F, Papahadjopoulos D (1981) Liposomes: preparation and characterisation In: Liposomes: From Physical Structure to Therapeutic Applications, ed: Knight C G. Elsevier, Amsterdam, p 51

Taberner D A, Norman C, Poller L, Burselm R W, Jones J B (1979) Br J Haematol 43 317

Tans G, van Zutphen H, Comfurius P, Hemker H C, Zwaal R F A (1979) Eur J Biochem 95 449

Tans G, Griffin J H (1982) Blood 59 69

Tappel A L (1953) Arch Biochem Biophys 44 378

Tappel A L (1955) J Am Oil Chem Soc 32 252

Tappel A L (1973) Fed Proc 32 1870

Teien A N, Lie M, Abildgaard U (1976) Thromb Res 8 413

Thackrah (1819) An Inquiry into the Nature and Properties of the Blood. Cox and Sons, London

Thiagarajan P, Shapiro S S, De Marco L (1980) J Clin Invest 66 397

Thiagarajan P, Shapiro S S (1983) In: Methods in Haematology 7 Disorders of thrombin formation. Churchill Livingstone, Edinburgh, p 101

Thomson J M (1980) Blood Coagulation and Haemostasis: A Practical Guide. Churchill Livingstone, Edinburgh, p 173

Thomson J M, Stevenson K J, Tomenson J A (1983) Report on proposed second WHO international reference thromboplastin, human plain to replace the WHO international reference preparation, human combined. Submitted to WHO

Thomson J M, Tomenson J A, Poller L (1984) Thromb Haemostas 52 336

Thomson J M, Poller L (1985) The activated partial thromboplastin time. In: Blood Coagulation and Haemostasis: a practical guide, 3rd edition, ed: Thomson J M. Churchill Livingstone, Edinburgh, 1985, p 301

Thomson J M, Darby K V, Poller L (1986) Thromb Haemostas 55 379

Thudichum J L W (1962) A treatise on the chemical constitution of the brain, ed: Drabkin D L. Archon, Hamden, Conn

Tilley L, Cribier S, Roelofsen B, Op den Kamp J A F, Van Deenen L L M (1986) FEBS Lett 194 21

Tipping P G, Campbell D A, Boyce N W, Holdworth S R (1988) Am J Pathol 131 206

Tomenson J A (1984) A Statistician's independent evaluation. In: Thromboplastin Calibration and Oral Anticoagulant Control, ed: van den Besselaar A M H P, Gralnick H R, Lewis S M. Martinus Nijhoff Publishers, Boston, p 87

Triggs W W (1944) Brit Pat No. 586693

Triplett D A, Harms C S, Koepke J A (1978) Am J Clin Path 70 556

Triplett D A, Brandt J T, Kaczor D, Schaeffer J (1983) Am J Clin Pathol 79 678

Troup S B, Reed C F (1958) J Clin Invest 37 937

Troup S B, Reed C F, Marinetti G V, Swisher S N (1960) J Clin Invest 39 342

Truglia J A, Stracher A (1981) Biochem Biophys Res Commun 100 814

Turner D L, Silver M J (1963) Nature 200 370

Van Deenen L L M, Houtsmuller U M T, De Haas G H, Molder E (1962) J Pharm Pharmacol 14 429

Van Deenen L L M (1981) FEBS Lett 123 3

Van den Besselaar A H M P (1988) Rev Iberoamer Thromb Haemostasia S1 19

Van Dieijen G, Tans G, Van Rijn J, Zwaal R F A, Rosing J (1981) Biochemistry 20 7096

Van Dieijen G, Tans G, Rosing J, Hemker H C (1981) J Biol Chem 256 3433

Van Dijck P W, De Kruijff B, Van Deenen L L M, De Gier J, Demel R A (1976) Biochim Biophys Acta 455 576

Van Meer G, Op Den Camp J A F (1982) J Cell Biochem 19 193

Van Rijn J L M L, Zwaal R F A, Hemker H C, Rosing H (1986) Haemostasis 16 216

Van Trotsenburg L (1975) Neurological Complications of Haemophilia. In: Handbook of Haemophilia, eds: Brinkhous K M, Hemker H C. Excerpta Medica, Amsterdam

Van de Waart P, Bruls H, Hemker H C, Lindhout T (1983) Biochemistry 22 2427

Varadi K, Hemker H C (1976) Thromb Res 8 303

Vehar G A, Davie E W (1980) Biochemistry 19 401

Verhallen P F J, Comfurius P, Bevers E M, Zwaal R F A (1986) Agents Action 20 181

Verkleij A J, Mombers C, Leunissen-Bijvelt J, Ververgaert P H J Th (1979) Nature 279 162

Walker F J, Sexton P W, Esmon C T (1979) Biochim Biophys Acta 571 303

Wallach D F H, Maurice P A, Steele B A, Surgenor D M (1959) J Biol Chem 234 2829

Wallin R, Patrick S D, Ballard J O (1986) Thromb Haemostas 55 235

Walsh P N (1978) Br J Haematol 40 311

Ware Branch D, Scott J, Kochenour N H, Hershgold E (1985) N Engl J Med 313 1322

Watts A, Marsh D, Knowles P F (1978) Biochemistry 17 1792

Wetherill G B (1981) Intermediate Statistical Methods. Chapman and Hall, London, p 13

White J G (1972) Am J Pathol 66 295

White D A (1973) The phospholipid composition of mammaliam tissues. In: Form and Function of Phospholipids, eds: Ansell G B, Hawthorne J N, Dawson R M C. Elsevier, Amsterdam, p 441

White G C (1980) Biochim Biophys Acta 631 130

WHO Expert Committee on Biol Stand (1977) 28th Report WHO Technical Report Series 610, WHO, Geneva

WHO Expert Committee on Biol Stand (1981) 31st Report WHO Technical Report Series 658, WHO, Geneva

WHO Expert Committee on Biol Stand (1983) 33rd Report WHO Technical Report Series 687, WHO Geneva

Wijngaards G, Hemker H C, van Deenen L L M (1977) Haemostasis 6 269

Wilcox J N, Smith K M, Schwartz S M, Gordon D (1989) Proc Natl Acad Sci USA 86 2839

Williams S W J (1964) J Biol Chem 239 933

Williams S W J (1966) J Biol Chem 241 1840

Woodside E E, Therriault D G, Kocholaty W (1964) Blood 24 76

Wooldridge I C (1893) On the Chemistry of the Blood and other Scientific Papers. Kegan Paul, London

Wu G S, Stein R A, Mead J F (1978) Lipids 13 517

Wu G S, Stein R A, Mead J F (1982) Lipids 17 403

Zeldis S, Nemerson Y, Pitlick F, Lentz T L (1972) Science 175 766

Zilversmit R D, Marcus A J, Ullman H L (1961) J Biol Chem 236 47

Zimmerman G A, McIntyre T M, Prescott S M (1985) Circulation 72 718

Zur M, Radcliffe R D, Oberdick J, Nemerson Y (1982) J Biol Chem 257 5623

Zwaal R F A, Roelofsen B, Colley C M (1973) Biochim Biophys Acta 300 159

Zwaal R F A, Roelofsen B, Comfurius P, van Deenen L L M (1975) Biochim Biophys Acta 406 83

Zwaal R F A, Comfurius P, van Deenen L L M (1977) Nature 268 358

Zwaal R F A, (1978) Biochim Biophys Acta 515 163

Zwaal R F A, Rosing J, Tans G, Bevers E M, Hemker H C (1980) Topological and kinetic aspects of phospholipids in blood coagulation. In: Regulation of Coagulation, eds: Mann K G, Taylor F B. Elsevier, New York, p 95

Zwaal R F A, Hemker H C (1982) Haemostasis 11 12

Zwaal R F A, Bevers E M (1983) Subcell Biochem 9 299

Zwaal R F A, Bevers E M (1988) Membrane phenomena and coagulant properties of platelets. In: Coagulation and Lipids, ed: Zwaal R F A. CRC Press, Boca Raton, p 203